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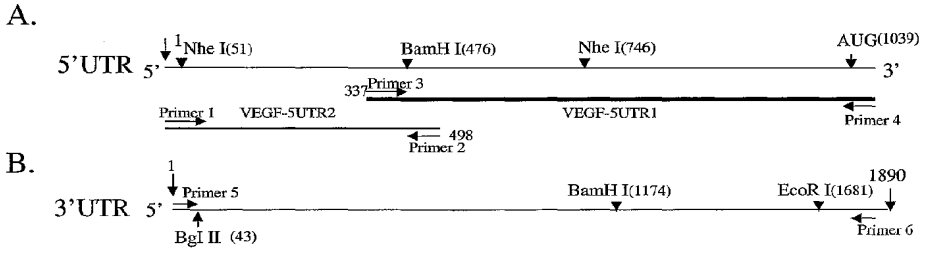

C.

Primer 1: 5'-AAAGTCGACGTAATCGCGGAGGCTTGGGCAGCCGG-3'
(Sal I; Stop code)

Primer 2: 5'-TTTGCGACTGGTCAGCTGCGGGATCCCAAG-3'

Primer 3: 5'-AAGTCGACGTAAGAGCTCCAGAGAGAAGTCGAG-3'
(Sal I; Stop code)

Primer 4: 5'-AAACCCGGGCAGCAAGGCAAGGCTCCAATGCAC-3'
(Xma I)

Primer 5: 5'-GCCGGGCAGGAGGAAGGAGCCTCCCTCAGGGTTTGGGGA-3'

Primer 6: 5'-CTGCACTAGAGACAAAGACGTGATGTTAAT-3'

(57) Abstract: The present invention relates to methods for identifying compounds that modulate untranslated region-dependent expression of a target gene. The invention particularly relates to using untranslated regions of a target gene or fragments thereof linked to a reporter gene to identify compounds that modulate untranslated region-dependent expression of a target gene. The methods of the present invention provide a simple, sensitive assay for high-throughput screening of libraries of compounds to identify pharmaceutical leads.

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METHODS FOR IDENTIFYING COMPOUNDS THAT MODULATE UNTRANSLATED REGION-DEPENDENT GENE EXPRESSION AND METHODS OF USING SAME

[0001] This application is entitled to and claims priority benefit to U.S. provisional application Serial No. 60/441,637, filed January 21, 2003, which is incorporated herein by reference in its entirety.

1. INTRODUCTION

[0002] The present invention relates to a method for screening and identifying compounds that modulate untranslated region-dependent expression of any gene. In particular, the invention provides reporter gene-based assays for the identification of compounds that modulate untranslated region-dependent expression of a gene. The methods of the present invention provide a simple, sensitive assay for high-throughput screening of libraries of compounds to identify pharmaceutical leads.

2. BACKGROUND OF THE INVENTION

2.1. Gene Expression

[0003] Every living organism is a product of expression of its genes in response to a developmental program (encoded in the genome itself) and environmental factors. Gene expression can be defined as the conversion of the nucleotide sequence of a gene into the amino acid sequence of a protein or into the nucleotide sequence of a stable RNA.

[0004] In eukaryotes, gene expression begins in the nucleus with the transcription of a gene into a premessenger-RNA, also referred to as a primary transcript. While still in the nucleus, the pre-mRNA is extensively modified. Each primary transcript is capped at the 5' end, associates with hnRNP proteins to form messenger RNA-protein particles ("mRNPs"), acquires a polyadenylic acid tail at the 3' end, and undergoes splicing to remove introns. In addition, the nucleotide sequence of certain pre-mRNAs can be altered post-transcriptionally in a process known as RNA editing. Thus processed, the mature mRNA is exported to the cytoplasm. Upon export, mRNA dissociates from hnRNP proteins and binds a set of cytosol-specific mRNA-binding proteins. Once in the cytoplasm, the mRNA either immediately associates with ribosomes and templates for protein synthesis or is localized to discrete cellular foci to direct compartment-specific protein synthesis. Degradation of mRNA and protein, which occurs both in the nucleus and the cytoplasm, concludes the list of processes that comprise gene expression.

2.2. Post-transcriptional Gene Expression Regulation

[0005] Gene expression is very tightly regulated. To produce the desired phenotype, each gene must be expressed at a defined time and at a defined rate and amount. Extensive experimental evidence indicates that post-transcriptional processes such as mRNA decay, translation, and mRNA localization constitute major control points in gene expression.

[0006] An aberration in the expression of one or more genes can be the cause or a downstream effect of a disease or other abnormality. Understanding gene expression regulation mechanisms in the normal/healthy/wild-type cell/body and during pathology will permit rational therapeutic intervention.

[0007] Regulation of gene expression both at the mRNA stability and translation levels is important in cellular responses to development or environmental stimuli such as nutrient levels, cytokines, hormones, and temperature shifts, as well as environmental stresses like hypoxia, hypocalcemia, viral infection, and tissue injury (reviewed in Guhaniyogi & Brewer, 2001, Gene 265(1-2):11-23). Furthermore, alterations in mRNA stability have been causally connected to specific disorders, such as neoplasia, thalassemia, and Alzheimer's disease, (reviewed in Guhaniyogi & Brewer, 2001, Gene 265(1-2):11-23 and Translational Control of Gene Expression, Sonenberg, Hershey, and Mathews, eds., 2000, CSHL Press). In contrast, regulation of gene expression at the mRNA localization level is primarily used by the cell to create and maintain polarity (internal gradients of protein concentration) (reviewed in Translational Control of Gene Expression, Sonenberg, Hershey, and Mathews, eds., 2000, CSHL Press).

2.3. mRNA Untranslated Regions in Gene Expression Regulation

[0008] A typical mRNA contains a 5' cap, a 5' untranslated region ("5' UTR") upstream of a start codon, an open reading frame, also referred to as coding sequence, that encodes a stable RNA or a functional protein, a 3' untranslated region ("3' UTR") downstream of the termination codon, and a poly(A) tail. Most studied *cis*-dependent RNA-based gene expression regulation elements map to the 5' or 3' UTRs.

[0009] Examples of 5' UTR regulatory elements include the iron response element ("IRE"), internal ribosome entry site ("IRES"), upstream open reading frame ("uORF"), male specific lethal element ("MSL-2"), G-quartet element, and 5'-terminal oligopyrimidine tract ("TOP") (reviewed in Keene & Tenenbaum, 2002, Mol Cell 9:1161 and Translational Control of Gene Expression, Sonenberg, Hershey, and Mathews, eds., 2000, CSHL Press).

[0010] Examples of 3' UTR regulatory elements include AU-rich elements ("AREs"), Selenocysteine insertion sequence ("SECIS"), histone stem loop, cytoplasmic

polyadenylation elements ("CPEs"), nanos translational control element, amyloid precursor protein element ("APP"), translational regulation element ("TGE")/direct repeat element ("DRE"), bruno element ("BRE"), 15-lipoxygenase differentiation control element (15-LOX-DICE), and G-quartet element (reviewed in Keene & Tenenbaum, 2002, Mol Cell 9:1161).

[0011] The internal ribosome entry site ("IRES") is one of the 5' UTR-based *cis*-acting elements of post-transcriptional gene expression control. IRESes facilitate cap-independent translation initiation by recruiting ribosomes directly to the mRNA start codon. IRESes are commonly located in the 3' region of a 5' UTR and are, as recent work has established, frequently composed of several discrete sequences. IRESes do not share significant primary structure homology, but do form distinct RNA secondary and tertiary structures. Some IRESes contain sequences complementary to 18S RNA and therefore may form stable complexes with the 40S ribosomal subunit and initiate assembly of translationally competent complexes. A classic example of an "RNA-only" IRES is the internal ribosome entry site from Hepatitis C virus. However, most known IRESes require protein co-factors for activity. More than 10 IRES *trans*-acting factors ("ITAFs") have been identified so far. In addition, all canonical translation initiation factors, with the sole exception of 5' end cap-binding eIF4E, have been shown to participate in IRES-mediated translation initiation (reviewed in Vagner et al., 2001, EMBO reports 2:893 and Translational Control of Gene Expression, Sonenberg, Hershey, and Mathews, eds., 2000, CSHL Press).

[0012] AU-rich elements ("AREs") are 3' UTR-based regulatory signals. AREs are the primary determinant of mRNA stability and one of the key determinants of mRNA translation initiation efficiency. A typical ARE is 50 to 150 nucleotides long and contains 3 to 6 copies of AU_3A pentamers embebbed in a generally A/U-enriched RNA region. The AU_3A pentamers can be scattered within the region or can stagger or even overlap (see, *e.g.*, Chen et al., 1995, Trends Biol Sci 20:465). One or several AU_3A pentamers can be replaced by expanded versions such as AU_4A or AU_5A heptamers (see, *e.g.*, Wilkund et al., 2002, J Biol Chem 277:40462 and Tholanikunnel and Malborn, 1997, J Biol Chem 272:11471). Single copies of the AU_nA (where n = 3, 4, or 5) elements placed in a random sequence context are inactive. The minimal active ARE has the sequence $U_2AU_nA(U/A)(U/A)$ (where n = 3, 4, or 5) (see, *e.g.* Worthington et al., 2002, J Biol Chem, 277:48558-64). The activity of certain AU-rich elements in promoting mRNA degradation is enhanced in the presence of distal uridine-rich sequences. These U-rich elements do not affect mRNA stability when present alone and thus that have been termed "ARE enhancers" (see, *e.g.*, Chen et al., 1994, Mol. Cell. Biol. 14:416).

[0013] Most AREs function in mRNA decay regulation and translation initiation regulation by interacting with specific ARE-binding proteins ("AUBPs"). There are at least 14 known cellular proteins that bind to AU-rich elements. AUBP functional properties determine ARE involvement in one or both pathways. For example, ELAV/HuR binding to c-*fox* ARE inhibits c-*fos* mRNA decay (see, *e.g.*, Brennan & Steitz, 2001, Cell Mol Life Sci. 58:266), association of tristetraprolin with TNFα ARE dramatically enhances TNFα mRNA hydrolysis (see, *e.g.*, Carballo et al., 1998, Science 281:1001), whereas interaction of TIA-1 with the TNFα ARE does not alter the TNFα mRNA stability but inhibits TNFα translation (see, *e.g.*, Piecyk et al., 2000, EMBO J. 19:4154). Given its size, it is very likely that one copy of a typical ARE is capable of interacting with several AUBPs molecules. Therefore, it is contended that in the cell the competition of multiple AUBPs for the limited set of AUBP-binding sites in an ARE and the resulting "ARE proteome" determines the ARE regulatory output.

[0014] The mechanism of ARE-mediated mRNA decay is poorly understood. It has been established that mammalian mRNA degradation proceeds in 3' to 5' direction and that the first step is deadenylation by poly(A)-specific ribonuclease ("PARN"). Recent work indicates that following deadenylation a stable multi-ribonuclease complex, termed exosome, degrades the body of the message. Exosome alone is capable of initiating and accomplishing mRNA decay. However, the presence of certain AREs upregulates degradation efficiency. Available evidence suggests that AREs alone or bound by AUBPs help recruit exosome to the RNA (see, *e.g.*, Chen et al., 2001, Cell 107:451 and Mukherjee et al., 2002, EMBO J. 21:165).

[0015] It has been reported that degradation of some mRNAs depends on ongoing translation. Thus, the translation machinery can also serve as a ribonulease-recruiting or stabilizing AUBPs-removing entity. Supporting evidence indicates that this mechanism may operate only on a subset of mRNAs under special cell growth conditions (see, *e.g.*, Curatola et al., 1995, Mol. Cell. Biol. 15:6331; Chen et al., 1995, Mol. Cell. Biol. 15:5777; Koeller et al., 1991, Proc. Natl. Acad. Sci. 88:7778; Savant-Bhonsale et al., 1992, Genes Dev. 6:1927; and Aharon & Schneider, 1993, Mol. Cell. Biol. 13:1971).

[0016] The mechanism of ARE-dependent translation regulation is understood even less well than that of ARE-mediated mRNA decay. It is not clear how a 3' UTR-localized element can affect translation initiation, a process that takes place in the 5' UTR. One plausible explanation comes from recent work showing that most or all cytoplasmic mRNPs are circularized via eIF4F - poly(A)-binding protein ("PABP") interaction. This interaction

can bring AREs in the 3' UTR into close proximity to the translation initiation site (see, *e.g.*, Wells et al., 1998, Mol. Cell. 2:135).

[0017] Citation or identification of any reference in Section 2 of this application is not an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

[0018] The present invention provides methods for identifying a compound that modulates untranslated region-dependent expression of a target gene. In particular, the invention provides methods for identifying compounds that down-regulate the translation or the stability of an mRNA of a target gene that is associated with or has been linked to the onset, development, progression or severity of a particular disease or disorder, said compounds functioning, at least in part, by targeting one or more aspects of untranslated region-dependent expression of the target gene. The invention also provides methods for identifying compounds that upregulate the translation or the stability of an mRNA of a target gene whose expression is beneficial to a subject with a particular disease or disorder, said compounds functioning, at least, in part, by targeting one or more aspects of untranslated region-dependent expression of the target gene. The invention encompasses the use of the compounds identified utilizing the methods of the invention for modulating the expression of a target gene *in vitro* and *in vivo*. In particular, the invention encompasses the use of the compounds identified utilizing the methods of the invention for the prevention, treatment or amelioration of a disease or disorder or a symptom thereof.

[0019] The invention provides reporter gene-based assays for the identification of a compound that modulates untranslated region-dependent expression of a target gene. The reporter gene-based assays may be conducted by contacting a compound with a cell genetically engineered to express a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions of a target gene, and measuring the expression of said reporter gene. Alternatively, the reporter gene-based assays may be conducted by contacting a compound with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions of a target gene, and measuring the expression of said reporter gene. The alteration in reporter gene expression relative to a previously determined reference range or a control in such reporter-gene based assays indicates that a particular compound modulates untranslated region-dependent expression of a target gene. In a specific embodiment, a compound identified utilizing a reporter gene-based assay described herein alters the expression of the reporter gene by at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least

90%, at least 95%, or at least 99%, or at least 1.5 fold, at least 2 fold, at least 2.5 fold, at least 5 fold, at least 7.5 fold or at least 10 fold relative to a control (*e.g.*, PBS), the absence of a control or a previously determined reference range in an assay described herein or well-known in the art. In order to exclude the possibility that a particular compound is functioning solely by modulating the expression of a target gene in an untranslated region-independent manner, one or more mutations (*i.e.*, deletions, insertions, or nucleotide substitutions) may be introduced into the untranslated regions operably linked to a reporter gene and the effect on the expression of the reporter gene in a reporter gene-based assay described herein can be determined.

[0020] In one embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) expressing a nucleic acid comprising a reporter gene operably linked to two, three or more untranslated regions of said target gene in a cell; (b) contacting said cell with a member of a library of compounds; and (c) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent regulation of expression is identified if the expression of said reporter gene in the presence of a compound is altered as compared to the expression of said reporter gene in the absence of said compound or the presence of a control (*e.g.*, phosphate buffered saline ("PBS")). In an alternative embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) expressing a nucleic acid comprising a reporter gene operably linked two, three or more untranslated regions of said target gene in a cell; (b) contacting said cell with a member of a library of compounds; and (c) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression is identified if the expression of said reporter gene is altered in the presence of the compound relative to a previously determined reference range.

[0021] In another embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) expressing a nucleic acid consisting of a reporter gene operably linked to one, two, three or more untranslated regions of the target gene in a cell; (b) contacting said cell with a member of a library of compounds; and (c) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent regulation of expression is identified if the expression of said reporter gene in the presence of a compound is altered as compared to the expression of said reporter gene in the absence of said compound or the presence of a control (*e.g.*, PBS). In an alternative

embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) expressing a nucleic acid consisting of a reporter gene operably linked to one, two, three or more untranslated regions of the target gene in a cell; (b) contacting said cell with a member of a library of compounds; and (c) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression is identified if the expression of said reporter gene in the presence of the compound is altered relative to a previously determined reference range.

[0022] In another embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) contacting a member of a library of compounds with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression is identified if the expression of said reporter gene in the presence of a compound is altered as compared to the expression of said reporter gene in the absence of said compound or the presence of a control (*e.g.*, PBS). In an alternative embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) contacting a member of a library of compounds with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression of a target gene is identified if the expression of said reporter gene in the presence of a compound is altered relative to a previously determined reference range.

[0023] In another embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) contacting a member of a library of compounds with a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting a reporter protein translated from said reporter gene, wherein a compound that modulates untranslated region-dependent expression is identified if the expression of said reporter gene in the presence of a compound is altered as compared to the expression of said reporter gene in the absence of said compound or the presence of a control (*e.g.*, PBS). In an alternative embodiment, the invention provides a method for identifying a compound that modulates untranslated

region-dependent expression of a target gene, said method comprising: (a) contacting a member of a library of compounds with a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression is identified if the expression of said reporter gene in the presence of a compound is altered relative to a previously determined reference range.

[0024] The invention also provides methods of identifying compounds that upregulate untranslated region-dependent expression of a target gene utlitizing the reporter gene-based assays described herein. In a specific embodiment, the invention provides a method of upregulating untranslated region-dependent expression of a target gene, said method comprising (a) contacting a compound with a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that upregulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of a compound is increased relative to the absence of the compound or a previously determined reference range. In another embodiment, the invention provides a method of upregulating untranslated region-dependent expression of a target gene, said method comprising (a) contacting a compound with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that upregulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of a compound is increased relative to the absence of the compound or a previously determined reference range.

[0025] The invention also provides methods of identifying compounds that down-regulate untranslated region-dependent expression of a target gene utlitizing the reporter gene-based assays described herein. In a specific embodiment, the invention provides a method of down-regulating untranslated region-dependent expression of a target gene, said method comprising (a) contacting a compound with a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that down-regulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of a compound is decreased relative to the absence of the compound or a previously determined reference range. In

another embodiment, the invention provides a method of down-regulating untranslated region-dependent expression of a target gene, said method comprising (a) contacting a compound with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that down-regulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of a compound is decreased relative to the absence of the compound or a previously determined reference range.

[0026] In accordance with the invention, the step of contacting a compound with a cell, or cell-free translation mixture and a nucleic acid in the reporter gene-based assays described herein is preferably conducted in an aqueous solution comprising a buffer and a combination of salts. In a specific embodiment, the aqueous solution approximates or mimics physiologic conditions. In another specific embodiment, the aqueous solution further comprises a detergent or a surfactant.

[0027] The present invention provides methods of identifying environmental stimuli (*e.g.*, exposure to different concentrations of CO_2 and/or O_2, stress, temperature shifts, and different pHs) that modulate untranslated region-dependent expression of a target gene utilizing the reporter gene-based assays described herein. In particular, the invention provides a method of identifying an environmental stimulus, said method comprising (a) contacting a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene with an environmental stimulus; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that modulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of an environmental stimuli is altered relative to the absence of the compound or a previously determined reference range. In a specific embodiment, the environmental stimuli is not hypoxia. In another embodiment, the environmental stimuli does not include a compound.

[0028] The reporter gene constructs utilized in the reporter gene-based assays described herein may comprise a 5' untranslated region ("UTR") of a target gene, a 3' UTR of a target gene, or a 5' UTR and a 3' UTR of a target gene operably linked to a reporter gene. In a specific embodiment, a reporter gene construct utilized in the reporter gene-based assays described herein comprises a 5' UTR of a target gene with a stable hairpin secondary structure operably linked to a reporter gene. In a preferred embodiment, a reporter gene construct utilized in the reporter gene-based assays described herein comprises a 5' UTR and a 3' UTR of a target gene. The untranslated regions of a target gene utilized to

construct a reporter gene construct may comprise one or more of the following elements: an iron response element ("IRE"), Internal ribosome entry site ("IRES"), upstream open reading frame ("uORF"), male specific lethal element ("MSL-2"), G quartet element, 5'-terminal oligopyrimidine tract ("TOP"), AU-rich element ("ARE"), selenocysteine insertion sequence ("SECIS"), histone stem loop, cytoplasmic polyadenylation element ("CPE"), nanos translational control element, amyloid precursor protein element ("APP"), translational regulation element ("TGE")/direct repeat element ("DRE"), Bruno element ("BRE"), and a 15-lipoxygenase differentiation control element ("15-LOX-DICE").

[0029] In addition to untranslated regions, the reporter gene constructs utilized in the reporter gene-based assays described herein may comprise one, two, three or more introns within the open reading frame ("ORF") of the reporter gene. Further, the 3' end of a reporter gene may be polyadenylated and/or the 5' end may be capped. In a specific embodiment, the 5' end of the reporter gene is not capped.

[0030] The reporter gene constructs utilized in the reporter gene-based assays described herein may comprise an untranslated region of a gene whose expression is associated with or has been linked to the onset, development, progression or severity of a particular disease or disorder. Alternatively, the reporter gene constructs utilized in the reporter gene-based assays described herein may comprise an untranslated region of a gene whose expression is beneficial to a subject with a particular disease or disorder. Examples of genes from which the untranslated regions may be obtained include, but are not limited, the gene encoding tumor necrosis factor alpha ("TNF-a"), the gene encoding granulocyte-macrophage colony stimulating factor ("GM-CSF"), the gene encoding granulocyte colony stimulating factor ("G-CSF"), the gene encoding interleukin 2 ("IL-2"), the gene encoding interleukin 6 ("IL-6"), the gene encoding vascular endothelial growth factor ("VEGF"), the genome encoding hepatitis C virus ("HCV"), the gene encoding survivin, or the gene encoding Her-2. In a specific embodiment, an untranslated region is obtained or derived from Her-2 and/or VEGF. In another embodiment, an untranslated region is not obtained or derived from the gene encoding Her-2. In another embodiment, an untranslated region is not obtained or derived from the gene encoding VEGF. In another embodiment, an untranslated region is not obtained or derived from the genes encoding VEGF and Her-2.

[0031] Any reporter gene well-known to one of skill in the art may be utilized in the reporter gene constructs described herein. Examples of reporter genes include, but are not limited to, the gene encoding firefly luciferase, the gene coding renilla luciferase, the genes encoding click beetle luciferase, the gene encoding green fluorescent protein, the gene encoding yellow fluorescent protein, the gene encoding red fluorescent protein, the gene

encoding cyan fluorescent protein, the gene encoding blue fluorescent protein, the gene encoding beta-galactosidase, the gene encoding beta-glucoronidase, the gene encoding beta-lactamase, the gene encoding chloramphenicol acetyltransferase, and the gene encoding alkaline phosphatase.

[0032] The reporter gene-based assays described herein may be conducted in a cell genetically engineered to express a reporter gene or *in vitro* utilizing a cell-free translation mixture. Any cell or cell line of any species well-known to one of skill in the art may be utilized in accordance with the methods of the invention. Further, a cell-free translation mixture may be derived from any cell or cell line of any species well-known to one of skill in the art. Examples of cells and cell types include, but are not limited to, human cells (*e.g.*, HeLa cells and 293 cells), yeast, mouse cells (*e.g.*, cultured mouse cells), rat cells (*e.g.*, cultured rat cells), Chinese hamster ovary ("CHO") cells, Xenopus oocytes, cancer cells (*e.g.*, undifferentiated cancer cells), primary cells, reticulocytes, wheat germ, rye embryo, or bacterial cells.

[0033] The compounds utilized in the reporter gene-based assays described herein may be members of a library of compounds. In specific embodiment, the compound is selected from a combinatorial library of compounds comprising peptoids; random biooligomers; diversomers such as hydantoins, benzodiazepines and dipeptides; vinylogous polypeptides; nonpeptidal peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; carbohydrate libraries; and small organic molecule libraries. In a preferred embodiment, the small organic molecule libraries are libraries of benzodiazepines, isoprenoids, thiazolidinones, metathiazanones, pyrrolidines, morpholino compounds, or diazepindiones.

[0034] Once a compound that modulates untranslated region-dependent expression of a target gene is identified, the structure of the compound may be determined utilizing well-known techniques or by referring to a predetermined code. For example, the structure of the compound may be determined by mass spectroscopy, NMR, vibrational spectroscopy, or X-ray crystallography.

[0035] A compound identified in accordance with the methods of the invention may directly bind to an RNA transcribed from a target gene. Alternatively, a compound identified in accordance with the methods of invention may bind to one or more *trans*-acting factors (such as, but not limited to, proteins) that modulate untranslated region-dependent expression of a target gene. Further, a compound identified in accordance with the methods of invention may disrupt an interaction between the 5' UTR and the 3' UTR.

[0036] In a specific embodiment, a compound identified in accordance with the methods of the invention reduces the translation efficiency and/or stability of an mRNA transcribed from a target gene by at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99%, or at least 1.5 fold, at least 2 fold, at least 2.5 fold, at least 5 fold, at least 7.5 fold or at least 10 fold relative to a control (*e.g.*, PBS), the absence of a control or a previously determined reference range in an assay described herein or well-known in the art. In another embodiment, a compound identified in accordance with the methods of the invention reduces the translation efficiency and/or stability of an mRNA transcribed from a target gene by at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99%, or at least 1.5 fold, at least 2 fold, at least 2.5 fold, at least 5 fold, at least 7.5 fold or at least 10 fold relative to a control (*e.g.*, PBS), the absence of a control or a previously determined reference range in an assay described herein or well-known in the art.

[0037] A compound that modulates untranslated region-dependent expression in a reporter gene-based assay described herein may be subsequently tested in *in vitro* assays (*e.g.*, cell-free assays) or *in vivo* assays (*e.g.*, cell-based assays) well-known to one of skill in the art or described herein for the effect of said compound on the expression of the target gene from which the untranslated regions of the reporter gene construct were derived. Further, to assess the specificity of a particular compound's effect on untranslated region-dependent expression of a target gene, the effect of said compound on the expression of one or more genes (preferably, a plurality of genes) can be determined utilizing assays well-known to one of skill in the art or described herein. In a preferred embodiment, a compound identified utilizing the reporter gene-based assays described herein has a specific effect on the expression of only one gene or a group of genes within the same signaling pathway.

[0038] In a specific embodiment, the specificity of a particular compound for an untranslated region of a target gene is determined by (a) contacting the compound of interest with a cell containing a nucleic acid comprising a reporter gene operably linked to an UTR of a different gene; and (b) detecting a reporter gene protein translated from the reporter gene, wherein the compound is specific for the untranslated region of the target gene if the expression of said reporter gene in the presence of the compound is not altered or is not substantially altered relative to a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS). In

another embodiment, the specificity of a particular compound for an untranslated region of a target gene is determined by (a) contacting the compound of interest with a panel of cells, each cell in a different well of a container (*e.g.*, a 48 or 96 well microtiter plate) and each cell containing a nucleic acid comprising a reporter gene operably linked to an UTR of a different gene; and (b) detecting a reporter gene protein translated from the reporter gene, wherein the compound is specific for the untranslated region of the target gene if the expression of said reporter gene in the presence of the compound is not altered or is not substantially altered relative to a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS). In accordance with this embodiment, the panel may comprise 5, 7, 10, 15, 20, 25, 50, 75, 100 or more cells. In another embodiment, the specificity of a particular compound for an untranslated region of a target gene is determined by (a) contacting the compound of interest with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to an UTR of a different gene; and (b) detecting a reporter gene protein translated from the reporter gene, wherein the compound is specific for the untranslated region of the target gene if the expression of said reporter gene in the presence of the compound is not altered or is not substantially altered relative to a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS). As used herein, the term "not substantially altered" means that the compound alters the expression of the reporter gene or target gene by less than 20%, less than 15%, less than 10%, less than 5%, or less than 2% relative to a negative control such as PBS.

[0039] The invention provides for methods for treating, preventing or ameliorating one or more symptoms of a disease or disorder associated with the aberrant expression of a target gene, said method comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a compound, or a pharmaceutically acceptable salt thereof, identified according to the methods described herein. In one embodiment, the target gene is aberrantly overexpressed. In another embodiment, the target gene is expressed at an aberrantly low level. In particular, the invention provides for a method of treating or preventing a disease or disorder or ameliorating a symptom thereof, said method comprising administering to a subject in need thereof an effective amount of a compound, or a pharmaceutically acceptable salt thereof, identified according to the methods described herein, wherein said effective amount increases the expression of a target gene beneficial in the treatment or prevention of said disease or disorder. The invention also provides for a method of treating or preventing a disease or disorder or ameliorating a symptom thereof, said method comprising administering to a subject in need

thereof an effective amount of a compound, or a pharmaceutically acceptable salt thereof, identified according to the methods described herein, wherein said effective amount decreases the expression of a target gene whose expression is associated with or has been linked to the onset, development, progression or severity of said disease or disorder. In a specific embodiment, the disease or disorder is a proliferative disorder, an inflammatory disorder, an infectious disease, a genetic disorder, an autoimmune disorder, a cardiovascular disease, or a central nervous system disorder. In an embodiment wherein the disease or disorder is an infectious disease, the infectious disease can be caused by a fungal infection, a bacterial infection, a viral infection, or an infection caused by another type of pathogen.

[0040] The invention provides a method for identifying a compound that inhibits or reduces angiogenesis, said method comprising: (a) contacting a member of a library of compounds with a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of a target gene; and (b) detecting the expression of said reporter gene, wherein if a compound that reduces the expression of said reporter gene relative to a previously determined reference range, or to the expression of said reporter gene in the absence of said compound or in the presence of a control (*e.g.*, PBS) is detected in (b), then (c) contacting the compound with a tumor cell and detecting the proliferation of said tumor cell, so that if the compound reduces or inhibits the proliferation of the tumor cell, the compound is identified as a compound that inhibits or reduces angiogenesis. The invention provides a method for identifying a compound that inhibits or reduces angiogenesis, said method comprising: (a) contacting a cell-free translation mixture with a member of a library of compounds and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of a target gene; and (b) detecting the expression of said reporter gene, wherein if a compound that reduces the expression of said reporter gene relative to a previously determined reference range, or to the expression of said reporter gene in the absence of said compound or in the presence of a control (*e.g.*, PBS) is detected in (b), then (c) contacting the compound with a tumor cell and detecting the proliferation of said tumor cell, so that if the compound reduces or inhibits the proliferation of the tumor cell, the compound is identified as a compound that inhibits or reduces angiogenesis. In a specific embodiment, the compound is further tested in an animal model for angiogenesis by, *e.g.*, administering said compound to said animal model and verifying that angiogenesis is inhibited by said compound in said animal model. In a preferred embodiment, the target gene is VEGF. In another embodiment, the compound identified in accordance with the methods of the invention inhibits or reduces angiogenesis by at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least

50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%, or at least 1.5 fold, at least 2 fold, at least 2.5 fold, at least 5 fold, at least 7.5 fold, or at least 10 fold relative to a control (*e.g.*, PBS) in an assay described herein or well-known in the art.

[0041] The invention provides for a method for identifying a therapeutic agent for the treatment or prevention of cancer, or amelioration of a symptom thereof, said method comprising: (a) contacting a member of a library of compounds with a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of a target gene; and (b) detecting the expression of said reporter gene, wherein if a compound that reduces the expression of said reporter gene relative to a previously determined reference range, or the expression of said reporter gene in the absence of said compound or the presence of a control (*e.g.*, PBS) is detected in (b), then (c) contacting the compound with a cancer cell and detecting the proliferation of said cancer cell, so that if the compound reduces or inhibits the proliferation of the cancer cell, the compound is identified as a therapeutic agent for the treatment or prevention of cancer, or amelioration of a symptom thereof. The invention also provides for a method for identifying a therapeutic agent for the treatment or prevention of cancer, or amelioration of a symptom thereof, said method comprising: (a) contacting a cell-free translation mixture with a member of a library of compounds and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of a target gene; and (b) detecting the expression of said reporter gene, wherein if a compound that reduces the expression of said reporter gene relative to a previously determined reference range, or the expression of said reporter gene in the absence of said compound or the presence of a control (*e.g.*, PBS) is detected in (b), then (c) contacting the compound with a cancer cell and detecting the proliferation of said cancer cell, so that if the compound reduces or inhibits the proliferation of the cancer cell, the compound is identified as a therapeutic agent for the treatment or prevention of cancer, or amelioration of a symptom thereof. In a specific embodiment, the compound is further tested in an animal model for cancer by, *e.g.*, administering said compound to said animal model and verifying that the compound is effective in reducing the proliferation or spread of cancer cells in said animal model. In a preferred embodiment, the target gene is survivin.

[0042] In a specific embodiment, the invention provides for a method of identifying a therapeutic agent for the treatment or prevention of breast cancer, or amelioration of a symptom thereof, said method comprising: (a) contacting a member of a library of compounds with a cell containing a nucleic acid comprising a reporter gene operably linked

to one, two, three or more untranslated regions of a target gene; and (b) detecting the expression of said reporter gene, wherein if a compound that reduces the expression of said reporter gene relative a previously determined reference range, or the expression of said reporter gene in the absence of said compound or the presence of a control is detected in (b), then (c) contacting the compound with a breast cancer cell and detecting the proliferation of said breast cancer cell, so that if the compound reduces or inhibits the proliferation of the breast cancer cell, the compound is identified as a therapeutic agent for the treatment or prevention of breast cancer, or amelioration of a symptom thereof. In another embodiment, the invention provides for a method of identifying a therapeutic agent for the treatment or prevention of breast cancer, or amelioration of a symptom thereof, said method comprising: (a) contacting a cell-free translation mixture with a member of a library of compounds and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of a target gene; and (b) detecting the expression of said reporter gene, wherein if a compound that reduces the expression of said reporter gene relative to a previously determined reference range, or the expression of said reporter gene in the absence of said compound or the presence of a control is detected in (b), then (c) contacting the compound with a breast cancer cell and detecting the proliferation of said breast cancer cell, so that if the compound reduces or inhibits the proliferation of the breast cancer cell, the compound is identified as a therapeutic agent for the treatment or prevention of breast cancer, or amelioration of a symptom thereof. In accordance with these embodiments, the compound may be further tested in an animal model for breast cancer by, *e.g.*, administering said compound to said animal model and verifying that the compound is effective in reducing the proliferation or spread of breast cancer cells in said animal model. In a preferred embodiment, the target gene is Her-2.

[0043] The invention also provides methods for upregulating or downregulating the expression of a target gene utilizing a compound identified in accordance with the methods described herein. The upregulation or downregulation of a target gene is particularly useful *in vitro* when attempting to produce a protein encoded by said target gene for use as a therapeutic or prophylactic agent, or in experiments conducted to, *e.g.*, identify the function or efficacy of said protein. In particular, the invention provides a method of modulating the expression of a target gene, said method comprising contacting a cell with an effective amount of a compound or pharmaceutically acceptable derivative thereof, identified according to the methods described herein. In one embodiment, the cell is a eucaryotic cell. In another embodiment, the cell is a procaryotic cell.

[0044] The invention further provides methods for verifying or confirming the ability of a compound to modulate untranslated region-dependent expression of a target gene. The ability of a compound to modulate untranslated region-dependent expression of a target gene can be verified or confirmed utilizing any of the assays described herein to identify such a compound. In a first embodiment, the invention provides a method for verifying the ability of a compound to modulate untranslated region-dependent expression of a target gene, said method comprising: (a) expressing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene in a cell; (b) contacting said cell with a compound; and (c) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression is verified if the expression of said reporter gene in the presence of a compound is altered as compared to a previously determined reference range or the expression of said reporter gene in the absence of said compound or the presence of a control.

[0045] In a second embodiment, the invention provides a method for verifying the ability of a compound to modulate untranslated region-dependent expression of a target gene, said method comprising: (a) contacting a compound with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression is verified if the expression of said reporter gene in the presence of a compound is altered as compared to a previously determined reference range or the expression of said reporter gene in the absence of said compound or the presence of a control.

[0046] In a third embodiment, the invention provides a method for verifying the ability of a compound to modulate untranslated region-dependent expression of a target gene, said method comprising: (a) contacting a compound with a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression is verified if the expression of said reporter gene in the presence of a compound is altered as compared to a previously determined reference range or the expression of said reporter gene in the absence of said compound or the presence of a control.

3.1. Terminology

[0047] As used herein, the term "5' cap" refers to a methylated guanine cap, *e.g.*, a 7 methylguanosine (5'-5') RNA triphosphate, that is added to the 5' end of a pre-mRNA.

[0048] As used herein, the term "ARE" refers to an adenylate uridylate rich element in the the 3' UTR of a mRNA.

[0049] As used herein, the term "compound" refers to any agent or complex that is being tested for its ability to modulate untranslated region-dependent expression of a target gene, or any agent or complex identified by the methods described herein. Examples of compounds include, but are not limited to, proteins, polypeptides, peptides, peptide analogs (including peptides comprising non-naturally occurring amino acids, *e.g.*, D-amino acids, phosphorous analogs of amino acids, such as α-amino phosphoric acids and α-amino phosphoric acids, or amino acids having non-peptide linkages), nucleic acids, nucleic acid analogs such as phosphorothioates and PNAs, hormones, antigens, antibodies, lipids, fatty acids, synthetic or naturally occurring drugs, opiates, dopamine, serotonin, catecholamines, thrombin, acetylcholine, prostaglandins, organic molecules, pheromones, adenosine, sucrose, glucose, lactose and galactose.

[0050] As used herein, the term "CUG repeat" refers to a repeat of a cytosine-uracil-guanine triplet in the 3' UTR of a mRNA.

[0051] As used herein, the term "cytosine rich element" refers to cytosine-rich stability determinant sequences in the 3' UTR of a mRNA.

[0052] As used herein, the terms "disorder" and "disease" refer to a condition in a subject.

[0053] As used herein, the term "effective amount" refers to the amount of a compound which is sufficient to reduce or ameliorate the severity, duration and/or a disease or disorder or a symptom theroef, prevent the advancement of a disease or disorder, cause regression of a disease or disorder, prevent the recurrence, development, or onset of one or more symptoms associated with a disease or disorder, or enhance or improve the prophylactic or therapeutic effect(s) of another therapy (*e.g.*, prophylactic or therapeutic agent).

[0054] As used herein, the term "fragment" refers to a nucleotide sequence comprising an nucleic acid sequence of at least 5 contiguous nucleic acid residues, at least 10 contiguous nucleic acid residues, at least 15 contiguous nucleic acid residues, at least 20 contiguous nucleic acid residues, at least 25 contiguous nucleic acid residues, at least 40 contiguous nucleic acid residues, at least 50 contiguous nucleic acid residues, at least 60 contiguous nucleic acid residues, at least 70 contiguous nucleic acid residues, at least contiguous 80 nucleic acid residues, at least contiguous 90 nucleic acid residues, at least contiguous 100 nucleic acid residues, at least contiguous 125 nucleic acid residues, at least 150 contiguous nucleic acid residues, at least contiguous 175 nucleic acid residues, at least contiguous 200 nucleic acid residues, or at least contiguous 250 nucleic acid residues of the

nucleotide sequence of untranslated region of a target gene. In a specific embodiment, a fragment of a untranslated region of a target gene retains at least one element of the untranslated region (*e.g.*, an IRES).

[0055] As used herein, the term "target RNA" refers to an RNA of interest, *i.e.*, the RNA transcribed from a target gene or a gene of interest. In a preferred embodiment, the target RNA contains one or more untranslated regions, and more preferably, contains at least one element of the untranslated region (*e.g.*, an IRES).

[0056] As used herein, the term "host cell" includes a particular subject cell transformed or transfected with a nucleic acid molecule and the progeny or potential progeny of such a cell. Progeny of such a cell may not be identical to the parent cell transfected with the nucleic acid molecule due to mutations or environmental influences that may occur in succeeding generations or integration of the nucleic acid molecule into the host cell genome.

[0057] As used herein, the term "in combination" refers to the use of more than one therapies (*e.g.*, prophylactic and/or therapeutic agents). The use of the term "in combination" does not restrict the order in which therapies (*e.g.*,prophylactic and/or therapeutic agents) are administered to a subject with a particular disease or disorder. A first therapy (*e.g.*, a prophylactic or therapeutic agent such as, *e.g.*, a compound identified in accordance with the methods of the invention) can be administered prior to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapy (*e.g.*, a prophylactic or therapeutic agent such as, *e.g.*, a chemotherapeutic agent, an anti-inflammatory agent or a TNF-α antagonist) to a subject with a particular disease or disorder.

[0058] As used herein, the term "IRE" refers to an iron response element in the 5' UTR or 3' UTR of a mRNA.

[0059] As used herein, the term "IRES" refers to an internal ribosome entry site in the 5' UTR of a mRNA.

[0060] As used herein, the term "library" refers to a plurality of compounds. A library can be a combinatorial library, *e.g.*, a collection of compounds synthesized using combinatorial chemistry techniques, or a collection of unique chemicals of low molecular weight (less than 1000 daltons) that each occupy a unique three-dimensional space.

[0061] As used herein, the term "ORF" refers to the open reading frame of a mRNA, *i.e.*, the region of the mRNA that is translated into protein.

[0062] As used herein, the terms "non-responsive" and "refractory" describe patients treated with a currently available therapy (*e.g.*, a prophylactic or therapeutic agent) for a disease or disorder, which is not clinically adequate to relieve one or more symptoms associated with such disease or disorder. Typically, such patients suffer from severe, persistently active disease and require additional therapy to ameliorate the symptoms associated with their disease or disorder.

[0063] As used herein, the phrase "pharmaceutically acceptable salt(s)," includes, but is not limited to, salts of acidic or basic groups that may be present in compounds identified using the methods of the present invention. Compounds that are basic in nature are capable of forming a wide variety of salts with various inorganic and organic acids. The acids that can be used to prepare pharmaceutically acceptable acid addition salts of such basic compounds are those that form non-toxic acid addition salts, *i.e.*, salts containing pharmacologically acceptable anions, including but not limited to sulfuric, citric, maleic, acetic, oxalic, hydrochloride, hydrobromide, hydroiodide, nitrate, sulfate, bisulfate, phosphate, acid phosphate, isonicotinate, acetate, lactate, salicylate, citrate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate and pamoate (*i.e.*, 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. Compounds that include an amino moiety may form pharmaceutically acceptable salts with various amino acids, in addition to the acids mentioned above. Compounds that are acidic in nature are capable of forming base salts with various pharmacologically acceptable cations. Examples of such salts include alkali metal or alkaline earth metal salts and, particularly, calcium, magnesium, sodium lithium, zinc, potassium, and iron salts.

[0064] As used herein, the term "poly(A) tail" refers to a polyadenylic acid tail that is added to the 3' end of a pre-mRNA.

[0065] As used herein, the terms "prophylactic agent" and "prophylactic agents" refer to any agent(s) which can be used in the prevention of a particular disease or disorder. In certain embodiments, the term "prophylactic agent" refers to a compound identified in the screening assays described herein. In certain other embodiments, the term "prophylactic agent" does not refer a compound identified in the screening assays described herein.

[0066] As used herein, the phrase "prophylactically effective amount" refers to the amount of a therapy (*e.g.*, a prophylactic agent) which is sufficient to result in the

prevention of the development, recurrence or onset of a disease or disorder or one or more symptoms associated thereof.

[0067] As used herein, the terms "prevent", " preventing" and "prevention" refer to the prevention of the development, recurrence or onset of a disease or disorder or one or more symptoms thereof resulting from the administration of one or more compounds identified in accordance the methods of the invention or the administration of a combination of such a compound and a known therapy for a particular disease or disorder.

[0068] As used herein, the term "previously determined reference range" refers to a reference range for the expression and/or the activity of a reporter gene or a target gene by a particular cell or in a particular cell-free translation mixture. Each laboratory will establish its own reference range for each particular assay, each cell type and each cell-free translation mixture. In a preferred embodiment, at least one positive control and at least one negative control are included in each batch of compounds analyzed.

[0069] As used herein, the term "small molecules" and analogous terms include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e*,. including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, organic or inorganic compounds having a molecular weight less than about 100 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. Salts, esters, and other pharmaceutically acceptable forms of such compounds are also encompassed.

[0070] As used herein, the terms "subject" and "patient" are used interchangeably herein. The terms "subject" and "subjects" refer to an animal, preferably a mammal including a non-primate (*e.g.*, a cow, pig, horse, cat, dog, rat, and mouse) and a primate (*e.g.*, a monkey such as a cynomolgous monkey and a human), and more preferably a human. In one embodiment, the subject is refractory or non-responsive to current therapies for a disease or disorder (*e.g.*, viral infections, fungal infections, bacterial infections, proliferative diseases or inflammatory diseases). In another embodiment, the subject is a farm animal (*e.g.*, a horse, a cow, a pig, *etc.*) or a household pet (*e.g.*, a dog or a cat). In a preferred embodiment, the subject is a human.

[0071] As used herein, the term "synergistic" refers to a combination of a compound identified using one of the methods described herein, and another therapy (preferably, a therapy which has been or is currently being used to prevent or treat a particular disease or disorder) which is more effective than the additive effects of the therapies. A synergistic effect of a combination of therapies (*e.g.*, prophylactic or therapeutic agents) permits the use of lower dosages of one or more of the therapies and/or less frequent administration of said therapies to a subject with a particular disease or disorder. The ability to utilize lower dosages of a therapy (*e.g.*, prophylactic or therapeutic agent) and/or to administer said therapy less frequently reduces the toxicity associated with the administration of said therapy to a subject without reducing the efficacy of said therapies in the prevention or treatment of a particular disease or disorder. In addition, a synergistic effect can result in improved efficacy of therapies in the prevention or treatment of a particular disease or disorder. Finally, a synergistic effect of a combination of therapies (*e.g.*, prophylactic or therapeutic agents) may avoid or reduce adverse or unwanted side effects associated with the use of either therapy alone.

[0072] As used herein, the term "target gene" refers to a gene or nucleotide sequence encoding a protein or polypeptide of interest. In a preferred embodiment, the gene or nucleotide sequence comprises an untranslated region.

[0073] As used herein, a "target nucleic acid" refers to RNA, DNA, or a chemically modified variant thereof. In a preferred embodiment, the target nucleic acid is RNA. In a preferred embodiment, the target nucleic acid refers to the untranslated region of an mRNA, such as, but not limited to, a 5' UTR and a 3' UTR. In another embodiment, the target nucleic acid refers to an open reading frame of an mRNA. A target nucleic acid also refers to tertiary structures of the nucleic acids, such as, but not limited to loops, bulges, pseudoknots, guanosine quartets and turns. A target nucleic acid also refers to RNA elements such as, but not limited to, the HIV TAR element, internal ribosome entry site, instability elements, and adenylate uridylate-rich elements, which are described in Section 5.1. Non-limiting examples of target nucleic acids are presented in Section 5.1 and Section 6.

[0074] As used herein, the terms "therapeutic agent" and "therapeutic agents" refer to any agent(s) which can be used in the prevention, treatment, management or amelioration of one or more symptoms of a particular disease or disorder. In certain embodiments, the term "therapeutic agent" refers to a compound identified in the screening assays described herein. In other embodiments, the term "therapeutic agent" does not refer to a compound identified in the screening assays described herein.

[0075] As used herein, the term "therapeutically effective amount" refers to that amount of a therapy (*e.g.*, a therapeutic agent) sufficient to reduce the severity of a disease or disorder, reduce the duration of a disease or disorder, ameliorate of one or more symptoms of a disease or disorder, prevent advancement of a disease or disorder, cause regression of the disease or disorder, or to enhance or improve the therapeutic effect(s) of another therapeutic agent. In a specific embodiment, with respect to the treatment of cancer, a therapeutically effective amount refers to the amount of a therapy (*e.g.*, a therapeutic agent) that inhibits or reduces the proliferation of cancerous cells, inhibits or reduces the spread of tumor cells (metastasis), inhibits or reduces the onset, development or progression of one or more symptoms associated with cancer, or reduces the size of a tumor. Preferably,with respect to the treatment of cancer, a therapeutically effective of a therapy (*e.g.*, a therapeutic agent) reduces the proliferation of cancerous cells or the size of a tumor by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% relative to a control (*e.g.*, PBS) in an assay described herein or well-known in the art.

[0076] In another embodiment, with respect to the treatment of a viral infection, a therapeutically effective amount refers to the amount of a therapy (*e.g.*, a therapeutic agent)sufficient to reduce or inhibit the replication of a virus, inhibit or reduce the spread of the virus to other tissues or subjects, or ameliorate one or more symptoms associated with the viral infection. Preferably, with respect to a viral infection, a therapeutically effective amount of a therapy (*e.g.*, a therapeutic agent) reduces the replication or spread of a virus by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% relative to a control (*e.g.*, PBS) in an assay described herein or well-known in the art.

[0077] In another embodiment, with respect to the treatment of a fungal infection, a therapeutically effective amount refers to the amount of a therapy (*e.g.*, a therapeutic agent) sufficient to inhibit or reduce the replication of the fungus, inhibit or reduce the replication or spread of the fungus to other tissues or subjects, or ameliorate one or more symptoms associated with the fungal infection. Preferably,with respect to a fungal infection, a therapeutically effective amount of a therapy (*e.g.*, a therapeutic agent) reduces the spread of a fungus by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least

60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% relative to a control (*e.g.*, PBS) in an assay described herein or well-known in the art.

[0078] In another embodiment, with respect to the treatment of a bacterial infection, a therapeutically effective amount refers to the amount of a therapy (*e.g.*, a therapeutic agent) sufficient to inhibit or reduce the replication of the bacteria, to inhibit or reduce the replication or spread of the bacteria to other tissues or subjects, or ameliorate one or more symptoms associated with the bacterial infection. Preferably, with respect to a bacterial infection, a therapeutically effective amount of a therapy (*e.g.*, a therapeutic agent) reduces the spread of a bacteria by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% relative to a control (*e.g.*, PBS) in an assay described herein or well-known in the art.

[0079] In another embodiment, with respect to the treatment of an inflammatory disorder, a therapeutically effective amount refers to the amount of a therapy (*e.g.*, a therapeutic agent) that reduces the inflammation of a joint, organ or tissue. Preferably, with respect to an inflammatory disorder, a therapeutically effective amount of a therapy (*e.g.*, a therapeutic agent) reduces the inflammation of a joint, organ or tissue by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% relative to a control (*e.g.*, PBS) in an assay described herein or well-known in the art.

[0080] As used herein, the terms "therapies" and "therapy" can refer to any protocol(s), method(s), and/or agent(s) that can be used in the prevention, treatment, management, or amelioration of a disease or disorder or one or more symptoms thereof.

[0081] As used herein, the terms "treat", "treatment" and "treating" refer to the reduction or amelioration of the progression, severity and/or duration of a disease or disorder or one or more symptoms thereof resulting from the administration of one or more compounds identified in accordance the methods of the invention, or the administration of a combination of therapies (*e.g.*, a compound identified in accordance with the methods of the invention and another therapeutic agent). In certain embodiments, such terms refer to the inhibition or reduction in the proliferation of cancerous cells, the inhibition or reduction the spread of tumor cells (metastasis), the inhibition or reduction in the onset, development or progression of one or more symptoms associated with cancer, or the reduction in the size of

a tumor. In other embodiments, such terms refer to the reduction or inhibition of the replication of a virus, the inhibition or reduction in the spread of a virus to other tissues or subjects, or the amelioration of one or more symptoms associated with a viral infection. In other embodiments, such terms refer to the reduction or inhibition of the replication of a fungus, the reduction or inhibition in the spread of a fungus to other tissues or subjects, or the amelioration of one or more symptoms associated with a fungal infection. In other embodiments, such terms refer to the inhibition or reduction of the replication of a bacteria, the inhibition or reduction in the spread of a bacteria to other tissues or subjects, or the amelioration of one or more symptoms associated with a bacterial infection. In other embodiments, such terms refer to a reduction in the swelling of one or more joints, organs or tissues, or a reduction in the pain associated with an inflammatory disorder.

[0082] As used herein, the term "UTR" refers to the untranslated region of a mRNA, *i.e.*, the region of the mRNA that is not translated into protein. In a preferred embodiment, the UTR is a 5' UTR, *i.e.*, upstream of the coding region, or a 3' UTR, *i.e.*, downstream of the coding region. In another embodiment, the term UTR corresponds to a reading frame of the mRNA that is not translated. In another embodiment, a UTR contains a fragment of an untranslated region of a mRNA. In a preferred embodiment, a UTR contains one or more regulatory elements that modulate untranslated region-dependent regulation of gene expression.

[0083] As used herein, the term "uORF" refers to an upstream open reading frame that is in the 5' UTR of the main open reading frame, *i.e.*, that encodes a functional protein, of a mRNA.

[0084] As used herein, the term "untranslated region-dependent expression" or "UTR-dependent expression" refers to the regulation of gene expression through untranslated regions at the level of mRNA expression, *i.e.*, after transcription of the gene has begun until the protein or the RNA product(s) encoded by the gene has degraded. In a preferred embodiment, the term "untranslated region-dependent expression" or "UTR-dependent expression" refers to the regulation of mRNA stability or translation. In a more preferred embodiment, the term "untranslated region-dependent expression" refers to the regulation of gene expression through regulatory elements present in an untranslated region(s).

4. DESCRIPTION OF DRAWINGS

[0085] FIGS. 1A-1B: Schematic representation of the VEGF 5'- and 3'-UTRs generated by PCR. A. VEGF 5'UTR was amplified from human genomic DNA by two separate PCR reactions. 5'UTR1, from position 337 to the 3' end plus first 45 nucleotides of VEGF open reading frame, was generated using primers 3 and 4. 5'UTR2, covered from

position 1 to 498, was generated with primers 1 and 2. In the overlap region of 5'UTR1 and 5'UTR2, the unique enzyme site BamH I was used to assemble the full length 5'UTR in the subsequent cloning. B. The full length VEGF 3'UTR was directly amplified from genomic DNA using primers 5 and 6. The two enzyme sites close to 5' end and 3' end of 3'UTR (BgI II and EcoR I) were used for subsequent cloning.

[0086] FIGS. 2A-2C: Identification of VEGF IRES domain in the VEGF mRNA 5'UTR. A. Dual luciferase vector used for mapping IRES function (Grentzmann et al., 1998, RNA 4:479-486). B. Schematic representation of the dicistronic plasmids used for transfection experiments. P2luc/vegf5utr1 is the dicistronic plasmid containing the VEGF 5'UTR1, in which nucleotides 337 to 1083 of the VEGF cDNA were fused to the firefly luciferase coding sequence; P2luc/vegf5utr-fl was generated by subcloning VEGF 5'UTR2 into the plasmid p2luc/vegf5utr1 between Sal I and BamH I; plasmid p2luc/vegf5'utr-delta51-476 is derived from p2luc/vegf5'utr-fl by removing the Nhe I fragment (nt 51 to 746); plasmid p2luc/vegf5utr-delta476-1038 was derived from p2luc/vegf5utr-fl by removing the sequence from BamH I site to the 3'end of 5'UTR; plasmid p2luc/vegf5utr-delta1-476 was derived from p2luc/vegf5utr-fl by removing the sequence from BamH I to the 5'end of 5'UTR. P2luc-e used as negative control in this study. C. The constructs depicted in panel A were transfected into 293T cells in the triplicate format and expression was analyzed by monitoring luciferase activity.

[0087] FIGS. 3A-3B: Generation of stable cell lines for cell based high throughput screening ("HTS"). A. Schematic representation of the monocistronic plasmid used in this study for generation of stable cell lines. B. Screening of stable cell lines. The plasmid depicted in panel A was transfected into 293T cells. 48 hours later, the transfected cells were seeded in 96 well plates at 100-500 cells per well and 200 mg/ml hygromycin was added for selection. The culture media plus hygromycin was changed every 3 to 4 days. After 2 weeks of selection, cells were screened under a microscope and single colony wells were expanded for further luciferase assays. The chart in panel shows the luciferase activities for 19 stable clones.

[0088] FIG. 4: Side by side comparison of luciferase activities for three stable clones (B9, D3 and H6). For each cell line, 5 x 10^5 cells per well were seeded in 24 well plate. 48 hours later, cells were lysed and assayed for luciferase activities. The luciferase activities were normalized against protein concentration.

[0089] FIG. 5: Sustained high expression of luciferase by B9 cells. B9 cells were continuously cultured in vitro for more than 3 months. At the time points indicated,

luciferase activity was tested with Promega's Bright Glow substrate and normalized against the protein concentration.

[0090] FIGS. 6A-6B: Reporter gene integration in B9 cells. The integration levels of the reporter gene were determined using semi-quantitative PCR. Series diluted plasmid pluc5'+3'vegf-UTR were included as positive control to make sure the reaction for sample (genomic DNA from B9 cells) was in the linear range, *i.e.*, not saturated. Panel A shows the PCR results for sample and positive control. The PCR band intensity for each reaction is at the bottom of the picture. Panel B shows the PCR standard curve, plotted with PCR band intensity against the amount of positive control plasmid loaded for PCR.

[0091] FIGS. 7A-7B: The 5' UTR of survivin can function as an internal ribosome entry site (IRES). A. Firefly luciferase assays on 293T cells transiently transfected with the survivin expression vectors in the absence of a stem-loop secondary structure. "5' + 3' UTR" represents the survivin expression vector containing the firefly luciferase reporter gene surrounded by both the 5' and 3' untranslated regions of survivin. "5' UTR" represents the survivin expression vector containing the firefly luciferase reporter gene preceded only by the 5' UTR of survivin. "3' UTR" represents the survivin expression vector containing the firefly luciferase reporter gene followed only by the 3' UTR of survivin. "no UTR" represents the survivin expression vector containing the firefly luciferase reporter gene lacking any surrounding untranslated regions of survivin. The survivin expression vectors were transiently transfected into 293T cells in duplicate (represented by the two bars for each construct in the graph) and firefly luciferase activity (measured in quadruplicate) was normalized to total protein concentration in each of the cell lysates. B. As in FIG. 7A, except that the survivin expression vectors containing the stem-loop secondary structure to separate cap-dependent from cap-independent translation were used.

[0092] FIGS. 8A-8C: Expression Vectors. A. Schematic representation of pCMR1, a high-level stable and transient mammalian expression vector designed to randomly integrate into the genome. B. Schematic representation of pMCP1, a high level stable and transient mammalian expression vector designed to site-specifically integrate into the genome of cells genetically engineered to contain the FRT site-specific recombination site via the Flp recombinase (see, *e.g.*, Craig, 1988, Ann. Rev. Genet. 22: 77-105; and Sauer, 1994, Curr. Opin. Biotechnol. 5: 521-527). C. Schematic representation of pCMR2, an episomal mammalian expression vector.

5. DETAILED DESCRIPTION OF THE INVENTION

[0093] The present invention provides methods for identifying compounds that modulate the untranslated region-dependent expression of any target gene. In particular, the invention provides simple, rapid and sensitive methods for identifying compounds that modulate untranslated region-dependent expression of a target gene utilizing reporter gene-based constructs comprising one or more mRNA untranslated regions ("UTRs") of the target gene. The reporter gene-based assays described herein can be utilized in a high throughput format to screen libraries of compounds to identify those compounds that modulate untranslated region-dependent expression of a target gene.

[0094] The reporter gene-based assays of the invention reduce the bias introduced by competitive binding assays which require the identification of use of a host cell factor (presumably essential for modulating RNA function) as a binding partner for the target RNA. The reporter gene-based assays of the invention are designed to detect any compound that modulates untranslated region-dependent expression of a target gene under physiologic conditions.

[0095] The reporter gene-based assays may be conducted by contacting a compound with a cell genetically engineered to express a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions (preferably, the 5' and/or 3' UTRs) of a target gene, and measuring the expression of said reporter gene. Alternatively, the reporter gene-based assays may be conducted by contacting a compound with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions of a target gene, and measuring the expression of said reporter gene. The alteration in reporter gene expression relative to a previously determined reference range or a control in such reporter-gene based assays indicates that a particular compound modulates untranslated region-dependent expression of a target gene. In order to exclude the possibility that a particular compound is functioning solely by modulating the expression of a target gene in an untranslated region-independent manner, one or more mutations (*i.e.*, deletions, insertions, or nucleotide substitutions) may be introduced into the untranslated regions operably linked to a reporter gene and the effect on the expression of the reporter gene in a reporter gene-based assay described herein can be determined.

[0096] The compounds identified in the reporter gene-based assays described herein that modulate untranslated region-dependent expression may be tested in *in vitro* assays (*e.g.*, cell-free assays) or *in vivo* assays (*e.g.*, cell-based assays) well-known to one of skill in the art or described herein for the effect of said compounds on the expression of the target gene from which the untranslated regions of the reporter gene construct were derived. Further, the specificity of a particular compound's effect on untranslated region-dependent

expression of one or more other genes (preferably, a plurality of genes) can be determined utilizing assays well-known to one of skill in the art or described herein. In a preferred embodiment, a compound identified utilizing the reporter gene-based assays described herein has a specific effect on the expression of only one gene or a group of genes within the same signaling pathway.

[0097] The structure of the compounds identified in the reporter gene-based assays described herein that modulate untranslated region-dependent expression can be determined utilizing assays well-known to one of skill in the art or described herein. The methods used will depend, in part, on the nature of the library screened. For example, assays or microarrays of compounds, each having an address or identifier, may be deconvoluted, *e.g.*, by cross-referencing the positive sample to an original compound list that was applied to the individual test assays. Alternatively, the structure of the compounds identified herein may be determined using mass spectrometry, nuclear magnetic resonance ("NMR"), X ray crystallography, or vibrational spectroscopy.

[0098] The invention encompasses the use of the compounds identified in accordance with the methods described herein for the modulation (*i.e.*, upregulation or downregulation) of the expression of a target gene. The upregulation or downregulation of a target gene is particularly useful *in vitro* when attempting to produce a protein encoded by said target gene for use as a therapeutic or prophylactic agent, or in experiments conducted to, *e.g.*, identify the function or efficacy of said protein. The invention also encompasses the use of the compounds identified in accordance with the methods described herein for the prevention, treatment or amelioration of a disease or disorder or a symptom thereof. Examples of diseases and disorders which may be prevented, treated or ameliorated utilizing a compound identified in accordance with the invention include, but are not limited to, proliferative disorders, disorders associated with aberrant angiogenesis, inflammatory disorders, infectious diseases, genetic disorders, autoimmune disorders, cardiovascular diseases, and central nervous system disorders. In an embodiment wherein the disease or disorder is an infectious disease, the infectious disease can be caused by a fungal infection, a bacterial infection, a viral infection, or an infection caused by another type of pathogen.

5.1. Untranslated Regions

[0099] Any untranslated region may be utilized in the reporter gene constructs described herein. An untranslated gene region(s) may be obtained or derived from a gene from any species, including, but not limited to, plants (*e.g.*, soybean, canola, cotton, wheat, corn, rice, potato, and tomato plants), viruses, bacteria, fungus and animals (including, but not limited to, mammals (primates and non-primates), farm animals (*e.g.*, horses, pigs,

cows, donkeys, etc.), pets (*e.g.*, guinea pigs, cats, and dogs), and humans). Untranslated regions may be obtained and the nucleotide sequence of the untranslated regions determined by any method well-known to one of skill in the art. The nucleotide sequence of an untranslated region for a target gene can be obtained, *e.g.*, from the literature or a database such as GenBank. Alternatively, the nucleotide sequence of the untranslated regions of a target gene may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid of an untranslated region of a target gene is not available, but the sequence of the untranslated region is known, a nucleic acid of the untranslated region may be chemically synthesized or obtained from a suitable source (*e.g.*, a cDNA library) by PCR amplification. Once the nucleotide sequence of an untranslated region is determined, the nucleotide sequence of the untranslated region may be manipulated using methods well-known in the art for the manipulation of nucleotide sequences, *e.g.*, recombinant DNA techniques, site directed mutagenesis, PCR, *etc.* (see, for example, the techniques described in Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel *et al.*, eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties), to generate an untranslated region having a different nucleotide sequence.

[00100] In one embodiment, an untranslated gene region(s) is obtained or derived from a gene whose expression is associated with or has been linked to the onset, development, progression or severity of a particular disease or disorder. In another embodiment, an untranslated gene region(s) is obtained or derived from a gene whose expression is beneficial to a subject with a particular disease or disorder. Examples of genes from which the untranslated regions may be obtained or derived from include, but are not limited to, cytokines, cytokine receptors, T cell receptors, B cell receptors, co-stimulatory molecules, clotting cascade factors, cyclins, cyclin inhibitors, oncogenes, growth factors, growth factor receptors, tumor suppressors, apoptosis inhibitor proteins, cell adhesion molecules, hormones, GTP-binding proteins, glycoproteins, ion channel receptors, calcium channel pumps, steroid receptors, opioid receptors, sodium channel pumps, heat shock proteins, MHC proteins, and tumor-associated antigens ("TAAs").

[00101] Specific examples of genes from which the untranslated regions may be obtained or derived from include, but are not limited, to the gene encoding abl, the gene encoding acetyl CoA carboxylase beta ("ACC2"; see, *e.g.*, OMIM accession number 601557, locus link accession number 32), the gene encoding acetylcholinesterase ("ACHE"; see, *e.g.*, OMIM accession number 100740, locus link accession number 43, GenBank

accession number NM 0006 65), the gene encoding actin, alpha cardiac ("ACTC"; see, *e.g.*, OMIM accession number 102540, locus link accession number 70), the gene encoding acyl-CoA dehydrogenase ("ACADVL"; see, *e.g.*, OMIM accession number 201475, locus link accession number 37), the gene encoding adiponectin ("ACRP30"; see, *e.g.*, OMIM accession number 605441, locus link accession number 9370, GenBank accession number NM 0047 97), the gene encoding ADP-ribosylation factor-4 ("ARF4"; see, *e.g.*, OMIM accession number 601177, locus link accession number 378, GenBank accession number NM 0017 ev 25), the gene encoding alpha-glucosidase, the gene encoding Alzheimer's disease amyloid A4 ("APP" or "A4" or "CVAP" or "AD1"; see, *e.g.*, OMIM accession number 104760, locus link accession number 351), the gene encoding angiogenin ("ANG" or "RNASE5"; see, *e.g.*, OMIM accession number 105850, locus link accession number 283, GenBank accession number NM 0011 45), the gene encoding angiopoietin1 ("ANG1"; see, *e.g.*, OMIM accession number 601667, locus link accession number 284), the gene encoding angiopoietin2 ("ANG2"; see, *e.g.*, OMIM accession number 601922, locus link accession number 285), the gene encoding angiostatin, the gene encoding angiotensin I-converting enzyme ("DCP1"; see, *e.g.*, OMIM accession number 106180, locus link accession number 1636), the gene encoding antigen CD82 ("KAI1"; see, *e.g.*, OMIM accession number 600623, locus link accession number 3732, GenBank accession number NM 0022 31), the gene encoding APC, the gene encoding atrial natriuretic factor, the gene encoding bactericidal/permeability-increasing protein ("BPI"; see, *e.g.*, OMIM accession number 109195, locus link accession number 671, GenBank accession number NM 0017 ev 25), the gene encoding bcl-2, the gene encoding beta-catenin ("CTNNB1"; see, *e.g.*, OMIM accession number 116806, locus link accession number 1499), the gene encoding beta-site APP-cleaving enzyme 2 ("BASE2"; see, *e.g.*, OMIM accession number 605668, locus link accession number 25825, GenBank accession number NM 1389 92), the gene encoding bile salt export pump ("ABCB1 1"; see, *e.g.*, OMIM accession number 603201, locus link accession number 8647), the gene encoding BMP, the gene encoding BNDF, the gene encoding bombesin receptor, the gene encoding brca1, the gene encoding brca2, the gene encoding C1q complement receptor (see, *e.g.*, OMIM accession number 120577, locus link accession number 22918), the gene encoding c-fms, the gene encoding c-myc, the gene encoding calcitonin, the gene encoding calcium-binding protein in macrophages ("MRP14"; see, *e.g.*, OMIM accession number 123886, locus link accession number 6280, GenBank accession number NM 0029 ev 65), the gene encoding calsenilin ("DREAM/CSEN" or "CREAM" or "KCh IP3"; see, *e.g.*, OMIM accession number 604662, locus link accession number 30818, GenBank accession number NM 0134), the gene encoding carnitine o-

palmitoyltransferase ("CPT2"; see, *e.g.*, OMIM accession number 600650, locus link accession number 1376), the gene encoding catechol-o-methyltransferase ("COMT"; see, *e.g.*, OMIM accession number 116790, locus link accession number 1312, GenBank accession number NM 000754, NM 007310), the gene encoding cathepsin K, the gene encoding CD40 ligand ("TNFSF5"; see, *e.g.*, OMIM accession number 300386, locus link accession number 959), the gene encoding cdk4 inhibitor, the gene encoding chemokine (C-C) receptor ("IL13R"; see, *e.g.*, OMIM accession number 601268, locus link accession number 1232), the gene encoding chemokine (C-X3-C) receptor 1 ("CX3CR1"; see, *e.g.*, OMIM accession number 601470, locus link accession number 1524), the gene encoding CLCA homolog ("hCLCA2"; see, *e.g.*, OMIM accession number 604003, locus link accession number 9635, GenBank accession number NM 0065 36), the gene encoding complement decay-accelerating factor ("DAF/CD55"; see, *e.g.*, OMIM accession number 125240, locus link accession number 1604), the gene encoding connective tissue growth factor ("CTGF"; see, *e.g.*, OMIM accession number 121009, locus link accession number 1490), the gene encoding corticotrophin releasing factor, the gene encoding CTLA4, the gene encoding cyclin D1, the gene encoding cyclin E, the gene encoding cyclin T1 (see, *e.g.*, OMIM accession number 602506, locus link accession number 904, GenBank accession number NM 0012 40), the gene encoding cyclin-dependent kinase inhibitor 1A ("p21" or "WAF1" or "CDKN1A" or "Cip1"; see, *e.g.*, OMIM accession number 116899, locus link accession number 1026, GenBank accession number NM 0784 67), the gene encoding cyclin-dependent kinase inhibitor 2A ("CDKN2A"; see, *e.g.*, OMIM accession number 600160, locus link accession number 1029), the gene encoding cystic fibrosis transmembrane conductance regulator ("CFTR"), the gene encoding cytochrome P-450, the gene encoding D-1 dopamine receptor ("DRD1"; see, *e.g.*, OMIM accession number 126449, locus link accession number 1812, GenBank accession number NM 00794, X589987), the gene encoding D-amino-acid oxidase ("DAO"; see, *e.g.*, OMIM accession number 124050, locus link accession number 1610, GenBank accession number NM 0019 17), the gene encoding damage specific DNA binding protein ("DDB1"; see, *e.g.*, OMIM accession number 600045, locus link accession number 1642), the gene encoding DCC, the gene encoding desmoglein 1 ("DSG1"; see, *e.g.*, OMIM accession number 125670, locus link accession number 1828), the gene encoding a dihydrofolate reductase ("DHFR"; see, *e.g.*, OMIM accession number 126060, locus link accession number 1719, GenBank accession number NM 0007 91), the gene encoding a disintegrin and metallo proteinase domain 33 ("ADAM 33"; see, *e.g.*, OMIM accession number 607114, locus link accession number 80332), the gene encoding DNA methyltransferase ("DNMT3b"; see, *e.g.*, OMIM

accession number 602900, locus link accession number 1789), the gene encoding DPP-IV, the gene encoding drebrin-1 dendritic spine protein ("DBN1"; see, *e.g.*, OMIM accession number 126660, locus link accession number 1627, GenBank accession number NM 004395, NM 080881), the gene encoding E-cadherin, the gene encoding effector cell protease receptor ("EPR1"; see, *e.g.*, OMIM accession number 603411, locus link accession number 8475), the gene encoding EGF, the gene encoding EGFR (see, *e.g.*, OMIM accession number 131550, locus link accession number 1956), the gene encoding an EGFR subunit, the gene encoding EIF4BP (see, *e.g.*, OMIM accession number 602223, locus link accession number 1978, GenBank accession number NM 0040 95), the gene encoding EMMPRIN (see, *e.g.*, OMIM accession number 109480, locus link accession number 682, GenBank accession number NM 0017 28), the gene encoding emotakin ATP-binding cassette, sub-family a, member 1 ("ABCA1"; see, *e.g.*, OMIM accession number 600046, locus link accession number 19), the gene encoding endostatin, the gene encoding eotaxin ("CCL11"; see, *e.g.*, OMIM accession number 601156, locus link accession number 6356, GenBank accession number NM 0029 86), the gene encoding erythropoietin ("EPO"; see, *e.g.*, OMIM accession number 133170, locus link accession number 2056, GenBank accession number NM 0007 99), the gene encoding estrogen receptor, the gene encoding factor IX, the gene encoding factor VIII, the gene encoding farnesyl transferase, the gene encoding FGF, the gene encoding FGF1 (see, *e.g.*, OMIM accession number 131220, locus link accession number 2246, GenBank accession number), the gene encoding FGF2 (see, *e.g.*, OMIM accession number 134920, locus link accession number 2247, GenBank accession number NM 0020 06), the gene encoding FGFR, the gene encoding fibrillin ("FBN1"; see, *e.g.*, OMIM accession number 134797, locus link accession number 2200), the gene encoding FMS-related tyrosine kinase 1 ("FLT1"; see, *e.g.*, OMIM accession number 165070, locus link accession number 2321, GenBank accession number NM 0020 ev 19), the gene encoding forkhead box C2 ("FOXC2"; see, *e.g.*, OMIM accession number 602402, locus link accession number 2303, GenBank accession number NM 0052 51), the gene encoding fos (see, *e.g.*, OMIM accession number 164810, locus link accession number 2353, GenBank accession number NM 0052 52), the gene encoding G-CSF, the gene encoding G-CSF 3 ("CSF3"; see, *e.g.*, OMIM accession number 138970, locus link accession number 1440), the gene encoding a GABA receptor, the gene encoding galanin ("GAL"; see, *e.g.*, OMIM accession number 137035, locus link accession number 2586), the gene encoding gastric inhibitory polypeptide ("GIP"; see, *e.g.*, OMIM accession number 137240, locus link accession number 2695), the gene encoding GDNF, the gene encoding GGF, the gene encoding GGRP, the gene encoding ghrelin ("GHRL"; see, *e.g.*, OMIM

accession number 605353, locus link accession number 51738), the gene encoding gip, the gene encoding glucagon, the gene encoding glucagon receptor ("GCGR"; see, *e.g.*, OMIM accession number 138033, locus link accession number 2642), the gene encoding glucagon-like peptide-1 ("GLP1"; see, *e.g.*, OMIM accession number 138030, locus link accession number 2641), the gene encoding glucokinase ("GCK"; see, *e.g.*, OMIM accession number 138079, locus link accession number 2645, GenBank accession number NM 0001 62), the gene encoding glutamic acid decarboxylase 2 (see, *e.g.*, OMIM accession number 138275), the gene encoding glutamic acid decarboxylase 3 (see, *e.g.*, OMIM accession number 138276), the gene encoding glutamic acid decarboxylase, brain, membrane form (see, *e.g.*, OMIM accession number 138277), the gene encoding glycogen synthase kinase-3A ("GSK-3A"; see, *e.g.*, OMIM accession number 606784, locus link accession number 2931), the gene encoding glycogen synthase kinase-3B ("GSK-3B"; see, *e.g.*, OMIM accession number 605004, locus link accession number 2932), the gene encoding GM-CSF (see, *e.g.*, OMIM accession number 138960, locus link accession number 1437), the gene encoding gonadotropin, the gene encoding gonadotropin releasing hormone, the gene encoding GRO2 oncogene or macrophage inflammatory protein-2-alpha precursor ("CXCL2"; see, *e.g.*, OMIM accession number 139110, locus link accession number 2920), the gene encoding growth hormone releasing factor, the gene encoding growth hormone, the gene encoding gsp, the gene encoding H-ras, the gene encoding heat shock protein ("HSP")-70, the gene encoding heparanase ("HPA"; see, *e.g.*, OMIM accession number 604724, locus link accession number 10855), the gene encoding hepatitis A virus cellular receptor ("HAVCR"; see, *e.g.*, OMIM accession number 606518, locus link accession number 26762), the gene encoding hepatitis B virus X interacting protein ("HBXIP"), the gene encoding hepsin ("HPN"; see, *e.g.*, OMIM accession number 142440, locus link accession number 3249, GenBank accession number NM 0021 51), the gene encoding Her-2 ("ERBB2"; see, *e.g.*, OMIM accession number 164870, locus link accession number 2064), the gene encoding HGF, the gene encoding histone acetyltransferase ("HAT1"; see, *e.g.*, OMIM accession number 603053, locus link accession number 8520, GenBank accession number NM 0036 42), the gene encoding histone deacetylase 1 ("HDAC1"; see, *e.g.*, OMIM accession number 601241, locus link accession number 3065), the gene encoding histone deacetylase 3 ("HDAC3"; see, *e.g.*, OMIM accession number 605166, locus link accession number 8841, GenBank accession number NM 0038 ev 83), the gene encoding HIV Tat Specific Factor 1 ("HTATSF1"; see, *e.g.*, OMIM accession number 300346, locus link accession number 27336), the gene encoding HMG CoA synthetase, the gene encoding HSP-90, the gene encoding huntingtin ("HD"; see, *e.g.*, OMIM accession number 143100,

locus link accession number 3064, GenBank accession number NM 0021 11), the gene encoding Hu antigen R ("HUR"; see, *e.g.*, OMIM accession number 603466, locus link accession number 1994, GenBank accession number NM 0014 19), the gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase ("HMGCR"; see, *e.g.*, OMIM accession number 142910, locus link accession number 3156), the gene encoding hypoxia-inducible factor 1 ("HIF-1A"; see, *e.g.*, OMIM accession number 603348, locus link accession number 3091), the gene encoding hypoxia-inducible factor 1-alpha inhibitor ("HIF1AN"; see, *e.g.*, OMIM accession number 606615, locus link accession number 55662), the gene encoding iduronate 2-sulfatase ("IDS"; see, *e.g.*, OMIM accession number 309900, locus link accession number 3423), the gene encoding IGF-1 (see, *e.g.*, OMIM accession number 147440, locus link accession number 3486), the gene encoding IGF-1R (see, *e.g.*, OMIM accession number 147370, locus link accession number 3480, GenBank accession number NM 0008 ev 75), the gene encoding IGF-2, the gene encoding IGF binding protein-2 ("IGFBP2"; see, *e.g.*, OMIM accession number 146731, locus link accession number 3485), the gene encoding IkB kinase ("IKBKB"; see, *e.g.*, OMIM accession number 603258, locus link accession number 3551), the gene encoding inositol pholyphosphate phosphatase-like 1 ("SHIP-2"; see, *e.g.*, OMIM accession number 600829, locus link accession number 3636, GenBank accession number NM 0015 67), the gene encoding insulin, the gene encoding interferon inducible protein ("CXCL10 (IP10)"; see, *e.g.*, OMIM accession number 147310, locus link accession number 3627, GenBank accession number NM 0015 65), the gene encoding interferon ("IFN")-α, the gene encoding interferon-α 1/13 precursor, the gene encoding interferon-α 5 precursor ("IFNA5"; see, *e.g.*, OMIM accession number 147565, locus link accession number 3442), the gene encoding interferon-α-16 precursor ("IFNA16"; see, *e.g.*, OMIM accession number 147580, locus link accession number 3449), the gene encoding IFN-β, the gene encoding IFN-β 1 ("IFNB1"; see, *e.g.*, OMIM accession number 147640, locus link accession number 3456), the gene encoding IFN-γ (see, *e.g.*, OMIM accession number 147440, locus link accession number 3479), the gene encoding insulin receptor ("INSR"; see, *e.g.*, OMIM accession number 147670, locus link accession number 3643, GenBank accession number NM 0002 08), the gene encoding interleukin-1 b ("IL1B"; see, *e.g.*, OMIM accession number 147720, locus link accession number 3553), the gene encoding interleukin-2 ("IL-2"; see, *e.g.*, OMIM accession number 147680, locus link accession number 3558), the gene encoding interleukin-3 ("IL-3"), the gene encoding interleukin-4 ("IL-4"; see, *e.g.*, OMIM accession number 147780, locus link accession number 3565, GenBank accession number NM 0005 89), the gene encoding interleukin-4 receptor ("IL4R"; see, *e.g.*, OMIM accession number 147781, locus link accession number

3566, GenBank accession number NM 0004 18), the gene encoding interleukin-5 ("IL-5"), the gene encoding interleukin-6 ("IL-6"; see, *e.g.*, OMIM accession number 147620, locus link accession number 3569), the gene encoding interleukin-7 ("IL-7"), the gene encoding interleukin-8 ("IL-8"; see, *e.g.*, OMIM accession number 146930, locus link accession number 3576), the gene encoding interleukin-9 ("IL-9"; see, *e.g.*, OMIM accession number 146931, locus link accession number 3578), the gene encoding interleukin-10 ("IL-10"; see, *e.g.*, OMIM accession number 124092, locus link accession number 3586, GenBank accession number NM 0005 72), the gene encoding interleukin-12 ("IL-12"), the gene encoding interleukin-12 beta chain precursor ("IL12B"; see, *e.g.*, OMIM accession number 161561, locus link accession number 3593), the gene encoding interleukin-13 ("IL-13"; see, *e.g.*, OMIM accession number 147683, locus link accession number 3596, GenBank accession number NM 0021 88), the gene encoding interleukin-15 ("IL-15"), the gene encoding interleukin-17F ("ML1"; see, *e.g.*, OMIM accession number 606496, locus link accession number 11274), the gene encoding interleukin-18 ("IL-18"; see, *e.g.*, OMIM accession number 600953, locus link accession number 3606), the gene encoding INI1/hSNF5 (see, *e.g.*, OMIM accession number 601607, locus link accession number 6598), the gene encoding jun, the gene encoding kallikrein 6 ("KLK6"; see, *e.g.*, OMIM accession number 602652, locus link accession number 5653, GenBank accession number NM 0027 74), the gene encoding KGF, the gene encoding ki-ras, the gene encoding kit ligand, stem cell factor ("KITLG (SCF)"; see, *e.g.*, OMIM accession number 184745, locus link accession number 4254, GenBank accession number NM 0008 99), the gene encoding klotho ("KL"; see, *e.g.*, OMIM accession number 604824, locus link accession number 9365, GenBank accession number NM 0047 95), the gene encoding L-myc, the gene encoding large tumor suppressor ("LATS1"; see, *e.g.*, OMIM accession number 603473, locus link accession number 9113, GenBank accession number NM 0046 ev 90), the gene encoding LDL receptor ("LDLR"; see, *e.g.*, OMIM accession number 606945, locus link accession number 3949, GenBank accession number NM 0005 27), the gene encoding leptin ("LEP"; see, *e.g.*, OMIM accession number 164160, locus link accession number 3952, GenBank accession number NM 0002 30), the gene encoding leptin receptor ("LEPR"; see, *e.g.*, OMIM accession number 601007, locus link accession number 3953), the gene encoding leucine amino peptidase-3 ("LAP3"; see, *e.g.*, OMIM accession number 606832, locus link accession number 51056), the gene encoding leukemia inhibitory factor ("LIF"; see, *e.g.*, OMIM accession number 159540, locus link accession number 3976), the gene encoding leukemia inhibitory factor receptor ("LIFR"; see, *e.g.*, OMIM accession number 151443, locus link accession number 3977), the gene encoding linker for activation

of T cells ("LAT"; see, *e.g.*, OMIM accession number 602354, locus link accession number 27040), the gene encoding livin (see, *e.g.*, OMIM accession number 605737, locus link accession number 79444, GenBank accession number NM 1393 ev 17), the gene encoding luteinizing hormone, the gene encoding luteinizing hormone releasing hormone, the gene encoding macrophage migration inhibitory factor ("MIF"; see, *e.g.*, OMIM accession number 153620, locus link accession number 4282, GenBank accession number NM 0024 15), the gene encoding major histocompatibility complex class I chain-related gene A ("MICA"; see, *e.g.*, OMIM accession number 600169, locus link accession number 4276, GenBank accession number NM 0002 47), the gene encoding major histocompatibility complex class I chain-related gene B ("MICB"; see, *e.g.*, OMIM accession number 602436, locus link accession number 4277, GenBank accession number NM 0059 31), the gene encoding matrix metalloproteinase 9 ("MMP9"; see, *e.g.*, OMIM accession number 120361, locus link accession number 4318), the gene encoding matrix metalloproteinase 12 ("MMP12"; see, *e.g.*, OMIM accession number 601046, locus link accession number 4321), the gene encoding max interacting protein 1 ("MXI1"; see, *e.g.*, OMIM accession number 600020, locus link accession number 4601), the gene encoding MCC, the gene encoding MDM2 (see, *e.g.*, OMIM accession number 164785, locus link accession number 4193, GenBank accession number NM 0023 92), the gene encoding METH-1, the gene encoding METH-2, the gene encoding methyl-CpG-binding endonuclease ("MBD4"; see, *e.g.*, OMIM accession number 603574, locus link accession number 8930, GenBank accession number NM 0039 ev 25), the gene encoding monoamine oxidase-A ("MAOA"; see, *e.g.*, OMIM accession number 309850, locus link accession number 4128, GenBank accession number NM 0002 ev 40), the gene encoding monoamine oxidase-B ("MAOB"; see, *e.g.*, OMIM accession number 309860, locus link accession number 4129), the gene encoding monocyte chemotactic protein 1 ("MCP1"; see, *e.g.*, OMIM accession number 158105, locus link accession number 6347), the gene encoding mos, the gene encoding MTS1, the gene encoding myc, the gene encoding myotrophin, the gene encoding N-acetyltransferase, the gene encoding N-cadherin, the gene encoding N-methyl D-aspartate ("NMDA") receptor, the gene encoding NAD(P)-dependent steroid dehydrogenase ("NSDHL"; see, *e.g.*, OMIM accession number 300275, locus link accession number 50814), the gene encoding natural resistance-associated macrophage protein ("NRAM P"; see, *e.g.*, OMIM accession number 600266, locus link accession number 6556), the gene encoding neural cell adhesion molecule 1 ("NCAM1"; see, *e.g.*, OMIM accession number 116930, locus link accession number 4684), the gene encoding neuron growth associated protein 43 ("GAP-43"; see, *e.g.*, OMIM accession number 162060, locus link accession number 2596),

the gene encoding NF1, the gene encoding NF2, the gene encoding NGF, the gene encoding a NGFR subunit, the gene encoding nm23, the gene encoding nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 ("NFKB1"; see, *e.g.*, OMIM accession number 164011, locus link accession number 4790), the gene encoding OSM, the gene encoding osteopontin ("OPN"; see, *e.g.*, OMIM accession number 166490, locus link accession number 6696), the gene encoding P-glycoprotein-1 ("PGY1"; see, *e.g.*, OMIM accession number 171050, locus link accession number 5243, GenBank accession number NM 0009 27), the gene encoding p38 MAP kinase ("p38" or "MAPK14"; see, *e.g.*, OMIM accession number 600289, locus link accession number 1432), the gene encoding p53, the gene encoding p300/CBP associated factor("PCAF"; see, *e.g.*, OMIM accession number 602303, locus link accession number 8850), the gene encoding parathyroid hormone, the gene encoding PDGF, the gene encoding PDGF, beta chain ("PDGF2"; see, *e.g.*, OMIM accession number 190040, locus link accession number 5155), the gene encoding a PDGFR subunit, the gene encoding peroxin-1 ("PEX1"; see, *e.g.*, OMIM accession number 602136, locus link accession number 5189), the gene encoding peroxisome assembly factor-2 ("PEX6"; see, *e.g.*, OMIM accession number 601498, locus link accession number 5190), the gene encoding peroxisome proliferator-activated receptor-gamma ("PPARg"; see, *e.g.*, OMIM accession number 601487, locus link accession number 5468), the gene encoding phenylalanine hydroxylase, the gene encoding phosphodiesterase, the gene encoding human phosphotyrosyl-protein phosphatase ("PTP-1B"; see, *e.g.*, OMIM accession number 176885, locus link accession number 5770, GenBank accession number NM 0028 27), the gene encoding placental growth factor ("PGF"; see, *e.g.*, OMIM accession number 601121, locus link accession number 5228, GenBank accession number NM 0026 ev 32), the gene encoding plasminogen activator inhibitor protein ("PAI1"; see, *e.g.*, OMIM accession number 173360, locus link accession number 5054), the gene encoding pleiotrophin ("PTN"; see, *e.g.*, OMIM accession number 162095, locus link accession number 5764), the gene encoding poly(rC) binding protein 2 ("PCBP2"; see, *e.g.*, OMIM accession number 601210, locus link accession number 5094), the gene encoding progranulin ("PCDGF" or "GRN"; see, *e.g.*, OMIM accession number 138945, locus link accession number 2896), the gene encoding prolactin ("PRL"; see, *e.g.*, OMIM accession number 176760, locus link accession number 5617, GenBank accession number NM 0009 48), the gene encoding proliferating cell nuclear antigen ("PCNA"; see, *e.g.*, OMIM accession number 176740, locus link accession number 5111), the gene encoding protein kinase B/Akt ("AKT1"; see, *e.g.*, OMIM accession number 164730, locus link accession number 207), the gene encoding protein kinase C, gamma ("PKCg"; see, *e.g.*, OMIM accession number 176980,

locus link accession number 5582), the gene encoding protein-tyrosine phosphatase, 4A, 3 ("PTP4A3"; see, *e.g.*, OMIM accession number 606449, locus link accession number 11156, GenBank accession number NM 0326 11), the gene encoding psoriasin ("PSOR1"; see, *e.g.*, OMIM accession number 600353, locus link accession number 6278, GenBank accession number NM 0029 63), the gene encoding ras, the gene encoding resistin ("Fizz3"; see, *e.g.*, OMIM accession number 605565, locus link accession number 56729, GenBank accession number NM 0204 15), the gene encoding retinoblastoma ("Rb"; see, *e.g.*, OMIM accession number 180200, locus link accession number 5925, GenBank accession number NM 0003 21), the gene encoding retinoblastoma 1 ("Rb1"), the gene encoding retinoblastoma-binding protein 1-like 1 ("RBBP1L1"; see, *e.g.*, locus link accession number 51742), the gene encoding 5-a reductase, the gene encoding ribonuclease/angiogenin inhibitor ("RNH"; see, *e.g.*, OMIM accession number 173320, locus link accession number 6050), the gene encoding S100 calcium-binding protein A8 ("MRP8"; see, *e.g.*, OMIM accession number 123885, locus link accession number 6279, GenBank accession number NM 0029 ev 64), the gene encoding signal transducer and activator of transcription 6 ("STAT6"; see, *e.g.*, OMIM accession number 601512, locus link accession number 6778), the gene encoding soluble-type polypeptide FZD4S ("FZD4S"; see, *e.g.*, OMIM accession number 604579, locus link accession number 8322), the gene encoding somatotrophin or somatotropin, the gene encoding src (see, *e.g.*, OMIM accession number 190090, locus link accession number 6714, GenBank accession number NM 0054 ev 17), the gene encoding survivin, the gene encoding T-cell lymphoma invasion and metastasis 1 ("TIAM1"; see, *e.g.*, OMIM accession number 600687, locus link accession number 7074), the gene encoding TEK tyrosine kinase ("TIE2"; see, *e.g.*, OMIM accession number 600221, locus link accession number 7010), the gene encoding telomerase, the gene encoding TGF-ß, the gene encoding TGF-ß1 (see, *e.g.*, OMIM accession number 190180, locus link accession number 7040), the gene encoding thrombomodulin ("THBD" or "THRM"; see, *e.g.*, OMIM accession number 188040, locus link accession number 7056), the gene encoding thrombopoietin ("THPO" or "TPO"; see, *e.g.*, OMIM accession number 600044, locus link accession number 7066), the gene encoding human trisosephosphate isomerase ("TPI1"; see, *e.g.*, OMIM accession number 109450, locus link accession number 7167), the gene encoding thyroid hormone, the gene encoding thyroid stimulating hormone, the gene encoding tissue factor, the gene encoding tissue inhibitor of metalloprotease 1 ("TIMP1"; see, *e.g.*, OMIM accession number 305370, locus link accession number 7076), the gene encoding tissue inhibitor of metalloprotease 2 ("TIMP2"; see, *e.g.*, OMIM accession number 188825, locus link accession number 7077, GenBank accession number NM 0032

55), the gene encoding tissue inhibitor of metalloprotease 4 ("TIMP4"; see, *e.g.*, OMIM accession number 601915, locus link accession number 7079, GenBank accession number NM 0032 56), the gene encoding TNF-a (see, *e.g.*, OMIM accession number 191160, locus link accession number 7124), the gene encoding troponin T ("TnT"), the gene encoding uncoupling protein 2 ("UCP2"; see, *e.g.*, OMIM accession number 601693, locus link accession number 7351, GenBank accession number NM 0033 55), the gene encoding urokinase plasminogen activator ("uPA"; see, *e.g.*, OMIM accession number 191840, locus link accession number 5328), the gene encoding utrophin ("UTRN"; see, *e.g.*, OMIM accession number 128240, locus link accession number 7402), the gene encoding v-myc myelocytomatosis viral oncogene homolog ("c-MYC"; see, *e.g.*, OMIM accession number 190080, locus link accession number 4609), the gene encoding vanilloid receptor subunit 1 ("VR1"; see, *e.g.*, OMIM accession number 602076, locus link accession number 7442, GenBank accession number NM 0187 ev 27, NM 08 0704, NM 0807 05, NM 0807 06), the gene encoding vascular endothelial growth factor ("VEGF"), the gene encoding virion infectivity factor ("VIF"), and the gene encoding VLA-4.

[00102] In a specific embodiment, an untranslated region is obtained or derived from the gene encoding Her-2. In another embodiment, an untranslated region is not obtained or derived from the gene encoding Her-2.

[00103] In one embodiment, an untranslated region is obtained or derived from the gene encoding VEGF. In another embodiment, an untranslated region is not obtained or derived from the gene encoding VEGF.

[00104] The untranslated regions may be obtained or derived from the genome of any virus utilizing any method well-known to one of skill in the art. The nucleotide sequence of an untranslated region for a genome of a virus can be obtained, *e.g.*, from the literature or a database such as GenBank. Examples of viruses from which the untranslated regions may be obtained or derived from include, but are not limited to, retrovirsues (*e.g.*, human immunodeficiency virus ("HIV") and human T cell leukemia virus ("HTLV"), herpesviruses (*e.g.*, herpes simplex virus, epstein barr virus and varicella zoster virus), reoviruses (*e.g.*, reovirus and rotavirus), picornaviruses (*e.g.*, poliovirus, rhinovirus and hepatitis A virus), togaviruses (*e.g.*, rubella virus), orthomyxovirus (*e.g.*,influenza virus), paramyxoviruses (*e.g.*, measles virus, mumps virus, respiratory syncytical virus and parainfluenza virus), filoviruses (*e.g.*, ebola virus and Marburg virus), rhabdoviruses (*e.g.*, rabies virus), coronaviruses (*e.g.*, coronavirus), rhinoviruses, hepatitis B virus, and hepatitis C virus.

[00105] The untranslated regions may be obtained or derived from the genome of any bacteria utilizing any method well-known to one of skill in the art. The nucleotide sequence of an untranslated region for a genome of a bacteria can be obtained, *e.g.*, from the literature or a database such as GenBank. Examples of bacteria from which the untranslated regions may be obtained or derived from include, but are not limited to, the *Aquaspirillum* family, *Azospirillum* family, *Azotobacteraceae* family, *Bacteroidaceae* family, *Bartonella* species, *Bdellovibrio* family, *Campylobacter* species, *Chlamydia* species (*e.g., Chlamydia pneumoniae*), clostridium, *Enterobacteriaceae* family (*e.g., Citrobacter* species, Edwardsiella, *Enterobacter aerogenes, Erwinia* species, *Escherichia coli,* Hafnia species, *Klebsiella* species, Morganella species, *Proteus vulgaris,* Providencia, *Salmonella* species, *Serratia marcescens,* and *Shigella flexneri*), *Gardinella* family, *Haemophilus influenzae, Halobacteriaceae* family, *Helicobacter* family, *Legionallaceae* family, *Listeria* species, *Methylococcaceae* family, mycobacteria (*e.g., Mycobacterium tuberculosis*), *Neisseriaceae* family, *Oceanospirillum* family, *Pasteurellaceae* family, *Pneumococcus* species, *Pseudomonas* species, *Rhizobiaceae* family, *Spirillum* family, *Spirosomaceae* family, *Staphylococcus* (*e.g.*, methicillin resistant *Staphylococcus aureus* and *Staphylococcus pyrogenes*), *Streptococcus* (*e.g., Streptococcus enteritidis, Streptococcus fasciae,* and *Streptococcus pneumoniae*), *VampirovibrHelicobacter* family, and *Vampirovibrio* family.

[00106] The untranslated regions may be obtained or derived from the genome of any fungus utilizing any method well-known to one of skill in the art. The nucleotide sequence of an untranslated region for a genome of a fungus can be obtained, *e.g.*, from the literature or a database such as GenBank. Examples of fungus from which the untranslated regions may be obtained or derived from include, but are not limited to, *Absidia* species (*e.g., Absidia corymbifera and Absidia ramosa*), *Aspergillus* species, (*e.g., Aspergillus flavus, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger,* and *Aspergillus terreus*), *Basidiobolus ranarum, Blastomyces dermatitidis,Candida* species (*e.g., Candida albicans, Candida glabrata, Candida kerr, Candida krusei, Candida parapsilosis, Candida pseudotropicalis, Candida quillermondii, Candida rugosa, Candida stellatoidea,* and *Candida tropicalis*), *Coccidioides immitis, Conidiobolus* species, *Cryptococcus neoforms, Cunninghamella* species, dermatophytes, *Histoplasma capsulatum, Microsporum gypseum, Mucor pusillus, Paracoccidioides brasiliensis, Pseudallescheria boydii, Rhinosporidium seeberi, Pneumocystis carinii, Rhizopus species* (*e.g., Rhizopus arrhizus, Rhizopus oryzae, and Rhizopus microsporus*), *Saccharomyces* species, *Sporothrix schenckii,* zygomycetes, and classes such as *Zygomycetes, Ascomycetes,* the *Basidiomycetes, Deuteromycetes,* and *Oomycetes.*

[00107] The untranslated regions may be obtained or derived from the genome of any plant utilizing any method well-known to one of skill in the art. The nucleotide sequence of an untranslated region for a genome of a plant can be obtained, *e.g.*, from the literature or a database such as GenBank, EMBL, DDBJ, rice genome database, cotton.genome database and maize genome database. Examples of plants from which the untranslated regions may be obtained or derived from include, but are not limited to, soybean, canola, cotton, corn, wheat, rice, tomato, and potato. Specific examples of plant genes from which an untranslated region may be obtained or derived from include, but are not limited to, triose phosphate, isomerase, fructose 1,6-bisphosphate adolase, fructose 1,6-bisphosphate, fructose 6-phosphate 2-kinase, phosphoglucoisomerase, pyrophsophate-dependent fructose-6-phosphate phosphotransferase, vacuolar H^+ translocating-pyrophosphate, invertase, sucrose synthase, hexokinase, fructokinase, NDP-kinase, glucose-6-phosphate 1-dehydrogenase, phosphoglucomutase, UDP-glucose pyrophosphorylase, glutenin genes, cis-prenyltransferase, lipoxygenase, and soybean vestitone reductase (see, *e.g.*, U.S. Patent Application Publication No. 2003/0135870 A1 and U.S. Patent Nos. 6,638,5252, 6,645,747, 6,627,797, and 6,617,493, which are incorporated herein by reference in its entirety).

[00108] In particular, a 5' UTR of a target gene, a 3' UTR of a target gene, or a 5' UTR and a 3' UTR of a target gene may be utilized in a reporter construct. In a specific embodiment, a 5' UTR of a target gene with a stable hairpin secondary structure is utilized in a reporter construct. In another specific embodiment, a reporter gene in the reporter construct contains an intron. In a preferred embodiment, a 5' UTR and a 3' UTR of a target gene are utilized in a reporter construct. In another preferred embodiment, a 5' UTR and a 3' UTR of a target gene and an intron-containing reporter gene are utilized in a reporter construct.

5.1.1. Elements of Untranslated Regions

[00109] Any element of an untranslated region(s) of a target gene may be utilized in the reporter gene constructs described herein. Elements of an untranslated region(s) may be obtained and the nucleotide sequence of the elements determined by any method well-known to one of skill in the art. The nucleotide sequence of an element of an untranslated region for a target gene can be obtained, *e.g.*, from the literature or a database such as GenBank. Alternatively, the nucleotide sequence of an element of an untranslated region of a target gene may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid of an element of an untranslated region of a target gene is not available, but the sequence of the element is known, a nucleic acid of the element may be chemically synthesized or obtained from a suitable source (*e.g.*, a cDNA library) by PCR

amplification. Once the nucleotide sequence of an element is determined, the nucleotide sequence of the element may be manipulated using methods well-known in the art for the manipulation of nucleotide sequences, *e.g.*, recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel *et al.*, eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties), to generate an element having a different nucleic acid sequence.

[00110] In one embodiment, an element(s) of an untranslated region comprises the full-length sequence of a UTR, *e.g.*, the 5' UTR or the 3' UTR. In a specific embodiment, an element(s) of an untranslated region that has been shown or has been suggested to be involved in the regulation of mRNA stability and/or translation is utilized in the reporter constructs described herein. Examples of elements of an untranslated region which may be utilized in the reporter constructs described herein include, but are not limited to, an IRE, IRES, uORF, MSL-2, G quartet element, 5'-terminal oligopyrimidine tract ("TOP"), ARE, SECIS, histone stem loop, CPE, nanos translational control element, APP, TGE/DRE, BRE, and a 15-LOX-DICE.

5.1.1.1. Iron Response Element

[00111] The maintenance of cellular iron homeostasis occurs at the level of mRNA stability and translation. Two components of this regulatory system have been defined: a *cis*-acting mRNA sequence/structure motif called an iron-responsive element ("IRE") and a specific *trans*-acting cytoplasmic binding protein, referred to herein as IRE-binding protein ("IRE-BP") (reviewed in, *e.g.*, Mikulits et al., 1999, Mutat Res. 437(3):219-30; Harrison & Arosio, 1996, Biochim Biophys Acta. 1275(3):161-203; Kuhn & Hentze, 1992, J Inorg Biochem. 1992, 47(3-4):183-95; and Harford & Klausner, 1990, Enzyme 44(1-4):28-41, the disclosures of which are hereby incorporated by reference in their entireties). Iron scarcity induces binding of IRE-BPs to a single IRE in the 5' UTR of ferritin, eALAS, aconitase, erythroid 5-aminolevulinic acid synthase, and SDHb mRNAs, which specifically suppresses translation initiation. Simultaneous interaction of IRE-BPs with multiple IREs in the 3' UTR of transferrin receptor mRNA selectively causes its stabilization. The pattern is reverted under iron overload: IRE-BP mRNA binding affinity is reduced, which results in efficient protein synthesis of target transcripts harboring IREs in the 5' UTR and rapid degradation of transferrin mRNA. Any gene containing an IRE including, but not limited to, the IREs described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.2. Internal Ribosome Entry Site

[00112] The internal ribosome entry site ("IRES") is one of the better characterized 5' UTR-based *cis*-acting elements of post-transcriptional gene expression control. IRESes facilitate cap-independent translation initiation by recruiting ribosomes directly to the 5' UTR of the mRNA. IRESes are commonly located in the 3' region of 5' UTR and are, as recent work has established, frequently composed of several discrete sequences. IRESes do not share significant primary structure homology, but do form distinct RNA tertiary structures. Some IRESes contain sequences complementary to 18S RNA and therefore may form stable complexes with 40S ribosomal subunit and initiate assembly of translationally competent complex. A classic example of an "RNA-only" IRES is the internal ribosome entry site from Hepatitis C virus. However, most known IRESes require protein co-factors for activity. More than 10 IRES trans-acting factors ("ITAFs") have been identified so far. In addition, all canonical translation initiation factors, with the sole exception of 5' end cap-binding eIF4E, have been shown to participate in IRES-mediated translation initiation (reviewed in Vagner et al., 2001, EMBO Reports 2:893 and Translational Control of Gene Expression, Sonenberg, Hershey, and Mathews, eds., 2000, CSHL Press, the disclosures of which are incorporated by reference in their entireties).

[00113] IRES were first identified in picornaviruses (see, *e.g.*, Pettetier & Sonenberg, 1988, Nature, 334:320-325). The 5' UTRs of all picornaviruses are long and mediate translational initiation by directly recruiting and binding ribosomes, thereby circumventing the initial cap-binding step. Although IRES elements are frequently found in viral mRNAs, they are rarely found in non-viral mRNAs. The non-viral mRNAs shown to contain functional IRES elements in their respective 5' UTRs include those encoding immunoglobulin heavy chain binding protein ("BiP") (see, *e.g.*, Macejak et al., 1991, Nature, 35390-4); Drosophila Antennapedia (see, *e.g.*, Oh et al., 1992, Genes Dev 6:1643-53) and Ultrabithorax (see, *e.g.*, Ye et al., 1997, Mol. Cell Biol. 17:1714-21); fibroblast growth factor 2 (see, *e.g.*, Vagner et al., 1995, Mol. Cell Biol. 15:35-44); initiation factor eIF4G (see, *e.g.*, Gan et al., 1998, J. Biol. Chem. 273:5006-12); proto-oncogene c-myc (see, *e.g.*, Nanbru et al., 1995, J. Biol. Chem. 272:32061-6 and Stoneley, 1998, Oncogene 16:423-8); vascular endothelial growth factor ("VEGF") (see, *e.g.*, Stein et al., 1998, Mol. Cell Biol. 18:3112-9), and X-linked inhibitor of apoptosis protein ("XIAP") (see, *e.g.*, U.S. Patent Nos. 6,159,709 and 6,171,821), the disclosures of which are incorporated by reference in their entireties. Any gene containing an IRES including, but not limited to, the IRESes described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.3. Male Specific Lethal Element

[00114] Male-specific expression of the protein male-specific-lethal 2 ("MSL-2") controls dosage compensation in Drosophila. MSL-2 protein is not produced in females and sequences in both the 5' and 3' UTRs are important for this sex-specific regulation because msl-2 gene expression is inhibited in females by Sex-lethal ("SXL"), an RNA binding protein known to regulate pre-mRNA splicing. An intron present in the 5' untranslated region of msl-2 mRNA contains putative SXL binding sites and is retained in female flies. The msl-2 pre-mRNA is alternatively spliced in a Sex-lethal-dependent fashion (see, *e.g.*, Gebauer et al., 1998, RNA 4(2):142-50 and Bashaw & Baker, 1995, Development 121(10):3245-58, the disclosures of which are hereby incorporated by reference in their entireties). Any gene containing an MSL-2 element including, but not limited to, the MSL-2 elements described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.4. G-quartet Element

[00115] A symmetrical structure of two tetrads of guanosine base pairs connected by three loops is commonly referred to as a "G-quartet", "G-quadruplex" or "G-tetraplex" structure (see, *e.g.*, Wang et al., 1993, Biochemistry 32:1899-1904; Macaya et al., 1993, Proc. Natl. Acad. Sci. 90:3745-3749; Schultze et al., 1994, J. Mol. Biol. 235:1532-1547; and Kelly et al., 1996, J. Mol. Biol. 256:417-422, the disclosures of which are incorporated by reference in their entireties). A G-quartet element was first identified as a conserved consensus sequence $GGNTGGN_{2\text{-}5}GGNTGG$ (SEQ ID NO: 1), which was present in single-stranded DNA aptamers that bind thrombin and inhibited thrombin-catalyzed fibrin-clot formation (see, *e.g.*, Bock et al., 1992, Nature 355:564-566, the dislosure of which is incorporated by reference in its entirety). A similar sequence in which the G-quartet structure is maintained when the length of the oligonucleotide between the G pairs is increased has been identified (see, *e.g.*, Dias et al., 1994, J. Am. Chem. Soc. 116:4479-4480, the disclosure of which is incorporated by reference in its entirety).

[0100] A G-quartet element has been identified in mRNAs associated with fragile X mental retardation syndrome (reviewed in, *e.g.*, Bardoni & Mandel, 2002, Curr Opin Genet Dev 12(3):284-93, the disclosure of which is incorporated by reference in its entirety). The fragile X mental retardation syndrome is caused by large methylated expansions of a CGG repeat in the FMR1 gene that lead to the loss of expression of FMRP, an RNA-binding protein. FMRP is proposed to act as a regulator of mRNA transport or translation that plays

a role in synaptic maturation and function and has been shown to interact preferentially with mRNAs containing a G quartet structure.

[0101] G-quartet oligonucleotides can have the sequence $GGN_xGGN_yGGN_zGG$ (SEQ ID NO: 2), wherein x, y and z indicate a variable number of nucleotides (see, *e.g.*, U.S. Patent No. 5,691,145, the disclosure of which is incorporated by reference in its entirety). While x, y and z are each typically at least about 2, preferably about 2-10, these segments may be longer if desired The regions of variable sequence (*i.e.*, $N_xN_yN_z$) are not critical in the present invention and can be varied in length and sequence without disrupting the characteristic G-quartet structure. As a general rule, the variable N sequences should not be self-complementary and should not contain G residues which would result in alternative G-quartet structures within the molecule. Representative G-quartet oligonucleotides are 15-20 nucleotides in length, but G-quartet oligonucleotides of any length which conform to the general formula $GGN_xGGN_yGGN_zGG$ (SEQ ID NO: 3) are also suitable. The G-quartet oligonucleotide is typically about 14-30 nucleotides in length. Any gene containing a G-quartet element including, but not limited to, the G-quartet elements described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.5. 5'-terminal Oligopyrimidine Tract

[0102] Translation control can be mediated by a terminal oligopyrimidine element ("TOP") present in the 5' untranslated region of ribosomal protein-encoding mRNAs. TOP elements adopt a specific secondary structure that prevents ribosome-binding and translation-initiation of ribsomal protein-encoding mRNAs. However, binding of cellular nucleic acid binding protein ("CNBP") or La proteins to the TOP hairpin structure abolishes the TOP-mediated transcription block and induces ribosomal protein production (see, *e.g.*, Schlatter & Fussenegger, 2003, Biotechnol Bioeng 81(1):1-12; Zhu et al., 2001, Biochim Biophys Acta 1521(1-3):19-29; and Crosio et al., 2000, Nucleic Acids Res. 28(15):2927-34, the disclosures of which are incorporated by reference in their entireties).

[0103] The immunosuppressant rapamycin selectively suppresses the translation of mRNAs containing a TOP tract adjacent to the cap structure. *Trans*-acting factors, some of which are regulated by rapamycin-responsive signaling pathways, that bind to the 5' untranslated region of TOP mRNAs may be involved in selective translational repression (see, *e.g.*, Kakegawa et al., 2002, Arch Biochem Biophys 402(1):77-83, the disclosure of which is incorporated by reference in its entirety). Any gene containing a TOP element including, but not limited to, the TOP elements described in the references cited above, can

be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.6. Adenylate Uridylate-rich Element

[0104] AU-rich elements ("AREs") are the most extensively studied 3' UTR-based regulatory signals. AREs are the primary determinant of mRNA stability and one of the key determinants of mRNA translation initiation efficiency.

[0105] A typical ARE is 50 to 150 nt long and contains 3 to 6 copies of AU_3A pentamer embedded in a generally A/U-enriched RNA region. The AU_3A pentamers can be scattered within the region or can stagger or even overlap (Chen et al., 1995, Trends Biol. Sciences 20:465, the disclosure of which is incorporated by reference in its entirety). One or several AU_3A pentamers can be replaced by expanded versions such as an AU_4A hexamer or AU_5A heptamer (see, *e.g.*, Wilkund et al., 2002, J. Biol. Chem. 277:40462 and Tholanikunnel & Malborn, 1997, J. Biol. Chem. 272:11471, the disclosures of which are incorporated by reference in their entireties). Single copies of the AU_nA (where n = 3, 4, or 5) elements placed in a random sequence context are inactive. The minimal active ARE has been determined to have the sequence $U_2AU_nA(U/A)(U/A)$ (where n = 3, 4, or 5) (see, *e.g.*, Worthington et al., 2002, J Biol Chem, 277:48558-64) the disclosure of which is incorporated by reference in its entirety). The activity of certain AU-rich elements in promoting mRNA degradation is enhanced in the presence of distal uridine-rich sequences. These U-rich elements do not affect mRNA stability when present alone and thus that have been termed "ARE enhancers" (see, *e.g.*, Chen et al., 1994, Mol. Cell. Biol. 14:416, the disclosure of which is incorporated by reference in its entirety).

[0106] Most AREs function in mRNA decay regulation and translation initiation regulation by interacting with specific ARE-binding proteins ("AUBPs"). There are at least 14 known cellular proteins that bind to AU-rich elements. AUBP functional properties determine ARE involvement in one or both pathways. For example, ELAV/HuR binding to c-fos ARE inhibits c-fos mRNA decay (see, *e.g.*, Brennan & Steitz, 2001, Cell Mol Life Sci. 58:266), association of tristetraprolin with TNFa ARE dramatically enhances TNFa mRNA hydrolysis (see, *e.g.*, Carballo et al., 1998, Science 281:1001), whereas interaction of TIA-1 with the TNFa ARE does not alter the TNFa mRNA stability but inhibits TNFa translation (see, *e.g.*, Piecyk et al., 2000, EMBO J. 19:4154).

[0107] Since AREs are clearly important in biological systems, including but not limited to a number of the early response genes that regulate cell proliferation and responses to exogenous agents, the identification of compounds that bind to one or more of the ARE clusters and potentially modulate the stability and translation of the target RNA can

potentially be of value as a therapeutic. Any gene containing an ARE including, but not limited to, the AREs described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.7. Selenocysteine Insertion Sequence

[0108] Selenium is an essential micronutrient that is now known to be incorporated as selenocysteine in a number of selenoproteins, glutathione peroxidase being the prototypical example. Selenocysteine is specifically encoded by the UGA codon, and inserted in peptide chains by a cotranslational mechanism that is able to override the normal function of UGA as a termination codon. In eukaryotes, efficient selenocysteine incorporation at UGA codons requires a cellular protein factor and a *cis*-acting structural signal usually located in the mRNA 3' untranslated region, consisting of a selenocysteine insertion sequence ("SECIS") in a characteristic stem-loop structure (see, *e.g.*, Peterlin et al., 1993, "Tat Trans-Activator" In Human Retroviruses; Cullen, Ed.; Oxford University Press: New York; pp. 75-100; Le & Maizel, 1989, J. Theor. Biol. 138:495-510; and reviewed in Hubert et al., 1996, Biochimie 78(7):590-6, the disclosures of which are incorporated by reference in their entireties). The required protein factor is presumed to be present in certain cells types that express selenoproteins, such as liver cells, lymphocytes, macrophages, thrombocytes, and other blood cells. In such cell types, the presence of a SECIS element in an mRNA is necessary and sufficient for in-frame UGA codons to be translated as selenocysteine.

[0109] A SECIS element is usually UAAAG, although other SECIS elements have been identified or variants have been constructed (see, *e.g.*, U.S. Patent Nos. 6,303,295, 5,849,520, and 5,700,660, the disclosures of which are incorporated by reference in their entireties). Any gene containing a SECIS element including, but not limited to, the SECIS elements described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.8. Histone Stem Loop

[0110] Replication-dependent histone mRNAs end with a conserved 26-nucleotide sequence that contains a 16-nucleotide stem-loop, *i.e.*, the histone stem loop, instead of a poly(A) tail. Formation of the 3' end of histone mRNA occurs by endonucleolytic cleavage of pre-mRNA releasing the mature mRNA from the chromatin template. Cleavage requires several *trans*-acting factors, including a protein, the stem-loop binding protein, which binds the 26-nucleotide sequence, and a small nuclear RNP, U7 snRNP (reviewed in, *e.g.*,

Dominski & Marzluff, 1999, Gene 239(1):1-14, the disclosure of which is incorporated by reference in its entirety).

[0111] Sequences of histone stem loops have been described in U.S. Patent Nos. 6,476,208; 6,455,280; 6,399,373; 6,346,381; 6,335,170; 6,331,396; 6,265,546; 6,265,167; 5,990,298; 5,908,779 and 5,843,770, the disclosures of which are incorporated by reference in their entireties. Any gene containing a histone stem loop including, but not limited to, the histone stem loops described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.9. Cytoplasmic Polyadenylation Element

[0112] Maturation-specific polyadenylation in Xenopus oocytes depends on the presence of a U-rich cytoplasmic polyadenylation element ("CPE") close to the 3' end of the RNA. RNAs that lack CPEs appear to be deadenylated by default when meiosis resumes. This default program also applies to maturing mouse oocytes (see, *e.g.*, Paynton & Bachvarova, 1994, Mol Reprod Dev 37(2):172-80, the disclosure of which is incorporated by reference in its entirety). CPEs have been identified in Wee1 protein tyrosine kinase mRNA (see, *e.g.*, Charlesworth et al., 2000, Dev Biol 227(2):706-19, the disclosure of which is incorporated by reference in its entirety), cyclin B1 mRNA (see, *e.g.*, Tay et al., 2000, Dev Biol 221(1):1-9 and Barkoff et al., 2000, Dev Biol 220(1):97-109, the disclosures of which are incorporated by reference in their entireties), and Xenopus Id3 mRNA (see, *e.g.*, Afouda et al., 1999, Mech Dev 88(1):15-31, the disclosure of which is incorporated by reference in its entirety).

[0113] A Xenopus oocyte CPE binding protein ("CPEB") binds the CPE and stimulates polyadenylation. CPEB is essential for the cytoplasmic polyadenylation of B4 RNA, G10, c-mos, cdk2, cyclins A1, B1 and B2 mRNAs which suggests that this protein is required for polyadenylation of most RNAs during oocyte maturation (see, *e.g.*, Stebbins-Boaz et al., 1996, EMBO J 15(10):2582-92, the disclosure of which is incorporated by reference in its entirety). Any gene containing a CPE including, but not limited to, the CPEs described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.10. Nanos Translational Control Element

[0114] The nanos translational control element is a discrete translational control element within the nanos 3' untranslated region that acts independently of the localization signal to mediate translational repression of unlocalized nanos RNA (see, *e.g.*, Clark et al.,

2002, Development 129(14):3325-34; Clark et al., 2000, Curr Biol 10(20):1311-4; Crucs et al., 2000, Mol Cell 5(3):457-67; Bergsten & Gavis, 1999, Development 126(4):659-69; Dahanukar & Wharton, 1996, Genes Dev (20):2610-20; and Gavis et al., 1996, Development 122(9):2791-800, the disclosures of which are incorporated by reference in their entireties).

[0115] During Drosophila embryogenesis, the Smaug protein represses translation of the nanos protein through an interaction with the nanos translational control element (see, *e.g.*, Green et al., 2002, Biochem Biophys Res Commun 297(5):1085-8, the disclosure of which is incorporated by reference in its entirety). Any gene containing a nanos translational control element including, but not limited to, the nanos translational control elements described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.11. Amyloid Precursor Protein Element

[0116] In one embodiment, the amyloid precursor protein element ("APP" element) refers to a novel iron-responsive element within the 5' untranslated region of the Alzheimer's amyloid precursor protein ("APP") transcript (+51 to +94 from the 5'-cap site) (see, *e.g.*, Rogers et al., 2002, J Biol Chem 277(47):45518-28). The APP mRNA IRE is located immediately upstream of an interleukin-1 responsive acute box domain (+101 to +146). The APP 5' UTR conferred translation was selectively down-regulated in response to intracellular iron chelation.

[0117] In another embodiment, the APP element refers to a 29 base instability element in the 3' UTR of the amyloid precursor protein involved in mRNA stability (see, *e.g.*, Westmark & Malter, 2001, Brain Res Mol Brain Res 90(2):193-201; Rajagopalan & Malter, 2000, J Neurochem 74(1):52-9; Amara et al., 1999, Brain Res Mol Brain Res 71(1):42-9; and Zaidi & Malter, 1995, J Biol Chem 270(29):17292-8, the disclosures of which are incorporated by reference in their entireties). Any gene containing a APP element including, but not limited to, the APP elements described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.12. Translation Regulation Element

[0118] Negative translational control elements in 3' UTRs regulate pattern formation, cell fate, and sex determination in a variety of organisms. tra-2 mRNA in Caenorhabditis elegans is required for female development but must be repressed to permit spermatogenesis in hermaphrodites. Translational repression of tra-2 mRNA in C. elegans

is mediated by tandemly repeated elements in its 3' UTR; these elements are called TGEs (for tra-2 and GLI element) (see, *e.g.*, Thompson et al., 2000, Mol Cell Biol 20(6):2129-37; Haag & Kimble, 2000, Genetics 155(1):105-16; and Jan et al., 1997, EMBO J 16(20):6301-13, the disclosures of which are incorporated by reference in their entireties). Any gene containing a TGE including, but not limited to, the TGEs described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.13. Direct Repeat Element

[0119] The direct repeat element ("DRE") is one control element in the 3' UTR of the tra-2 mRNA that causes repression of tra-2, *i.e.*, inhibits translation of tra-2 mRNA, which is responsible for the onset of hermaphrodite spermatogenesis in C. elegans (see, *e.g.*, Goodwin et al., 1993, Cell 75:329-339, the disclosure of which is incorporated by reference in its entirety). Three germline-specific regulators have been identified that mediate DRE regulation by the tra-2 3' UTR. These include DRFQ2/GLD-1, a protein that specifically binds the DRE (see, *e.g.*, Goodwin et al., 1993, Cell 75:329-339) and controls tra-2 translation (see, *e.g.*, Jan et al. 1999, EMBO J. 18:258-269); FOG-2, a protein that binds GLD-1 and is required for the onset of hermaphrodite spermatogenesis (see, *e.g.*, Schedl & Kimble, 1988, Genetics 119:43-61); and laf-1, a gene that has not yet been identified at the molecular level (see, *e.g.*, Goodwin et al., 1997, Development 124:749-758), the disclosures of which are incorporated by reference in their entireties. Any gene containing a DRE including, but not limited to, the DREs described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.14. Bruno Response Element

[0120] The Bruno Response Element ("BRE"), is located in the 3' untranslated region (UTR) of oskar mRNA (see, *e.g.*, Castagnetti et al., 2000, Development 127(5):1063-8, the disclosure of which is incorporated by reference in its entirety). The coupled regulation of oskar mRNA localization and translation in time and space is critical for correct anteroposterior patterning of the Drosophila embryo. Localization-dependent translation of oskar mRNA, a mechanism whereby oskar RNA localized at the posterior of the oocyte is selectively translated and the unlocalized RNA remains in a translationally repressed state, ensures that Oskar activity is present exclusively at the posterior pole. Genetic experiments indicate that translational repression involves the binding of Bruno protein to multiple sites, the BREs, in the 3' untranslated region of oskar mRNA. Any gene containing a BRE

including, but not limited to, the BREs described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.1.1.15. 15-lipoxygenase Differentiation Control Element

[0121] The translation of 15-lipoxygenase ("LOX") mRNA in erythroid precursor cells and of the L2 mRNA of human papilloma virus type 16 (HPV-16) in squamous epithelial cells is silenced when either of these cells is immature and is activated in maturing cells by unknown mechanisms. It has been shown that hnRNP K and the c-Src kinase specifically interact with each other, leading to c-Src activation and tyrosine phosphorylation of hnRNP K in vivo and in vitro. c-Src-mediated phosphorylation reversibly inhibits the binding of hnRNP K to the differentiation control element ("DICE") of the LOX mRNA 3' untranslated region in vitro and specifically derepresses the translation of DICE-bearing mRNAs in vivo (see, *e.g.*, Ostareck-Lederer et al., 2002, Mol Cell Biol 22(13):4535-43, the disclosure of which is incorporated by reference in its entirety).

[0122] Cytidine-rich 15-lipoxygenase differentiation control element ("15-LOX-DICE") is a multifunctional *cis*-acting element found in the 3' untranslated region of numerous eukaryotic mRNAs. It binds KH domain proteins of the type hnRNP E and K, thus mediating mRNA stabilization and translational control. Translational silencing is caused by formation of a simple binary complex between DICE and recombinant hnRNP E1. Electromobility shift assays and sucrose gradient centrifugation demonstrate that rabbit 15-LOX-DICE, which is composed of ten subunits of the sequence (CCCCPuCCCUCUUCCCCAAG, SEQ ID NO: 4), is able to bind up to ten molecules of hnRNP E1 (see, *e.g.*, Reimann et al., 2002, J Mol Biol 315(5):965-74 and Thiele et al., 1999, Adv Exp Med Biol 447:45-61, the disclosures of which are incorporated by reference in their entireties). Any gene containing a 15-LOX-DICE including, but not limited to, the 15-LOX-DICEs described in the references cited above, can be used in the present invention to identify compounds that modulate untranslated region-dependent gene expression.

5.2. Reporter Gene Constructs, Transfected Cells, and Cell-Free Extracts

[0123] The invention provides for specific vectors containing a reporter gene flanked by one or more UTRs of a target gene and host cells transfected with the vectors. The invention also provides for the *in vitro* translation of a reporter gene flanked by one or more UTRs of a target gene. Techniques for practicing this specific aspect of this invention will employ, unless otherwise indicated, conventional techniques of molecular biology,

microbiology, and recombinant DNA manipulation and production, which are routinely practiced by one of skill in the art. See, *e.g.*, Sambrook, 1989, Molecular Cloning, A Laboratory Manual, Second Edition; DNA Cloning, Volumes I and II (Glover, Ed. 1985); Oligonucleotide Synthesis (Gait, Ed. 1984); Nucleic Acid Hybridization (Hames & Higgins, Eds. 1984); Transcription and Translation (Hames & Higgins, Eds. 1984); Animal Cell Culture (Freshney, Ed. 1986); Immobilized Cells and Enzymes (IRL Press, 1986); Perbal, A Practical Guide to Molecular Cloning (1984); Gene Transfer Vectors for Mammalian Cells (Miller & Calos, Eds. 1987, Cold Spring Harbor Laboratory); Methods in Enzymology, Volumes 154 and 155 (Wu & Grossman, and Wu, Eds., respectively), (Mayer & Walker, Eds., 1987); Immunochemical Methods in Cell and Molecular Biology (Academic Press, London, Scopes, 1987), Expression of Proteins in Mammalian Cells Using Vaccinia Viral Vectors in Current Protocols in Molecular Biology, Volume 2 (Ausubel et al., Eds., 1991).

5.2.1. Reporter Genes

[0124] Any reporter gene well-known to one of skill in the art may be used in reporter gene constructs to ascertain the effect of a compound on untranslated region-dependent expression of a target gene. Reporter genes refer to a nucleotide sequence encoding a protein that is readily detectable either by its presence or activity. Reporter genes may be obtained and the nucleotide sequence of the reporter gene determined by any method well-known to one of skill in the art. The nucleotide sequence of a reporter gene can be obtained, *e.g.*, from the literature or a database such as GenBank. Alternatively, a polynucleotide encoding a reporter gene may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a particular reporter gene is not available, but the sequence of the reporter gene is known, a nucleic acid encoding the reporter gene may be chemically synthesized or obtained from a suitable source (*e.g.*, a cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from, any tissue or cells expressing the reporter gene) by PCR amplification. Once the nucleotide sequence of a reporter gene is determined, the nucleotide sequence of the reporter gene may be manipulated using methods well-known in the art for the manipulation of nucleotide sequences, *e.g.*, recombinant DNA techniques, site directed mutagenesis, PCR, (see, for example, the techniques described in Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel *et al.*, eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their

entireties), to generate reporter genes having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

[0125] Examples of reporter genes include, but are not limited to, luciferase (*e.g.*, firefly luciferase, renilla luciferase, and click beetle luciferase), green fluorescent protein ("GFP") (*e.g.*, green fluorescent protein, yellow fluorescent protein, red fluorescent protein, cyan fluorescent protein, and blue fluorescent protein), beta-galactosidase ("b-gal"), beta-glucoronidase, beta-lactamase, chloramphenicol acetyltransferase ("CAT"), and alkaline phosphatase ("AP"). In a preferred embodiment, a reporter gene utilized in the reporter constructs is easily assayed and has an activity which is not normally found in the cell or organism of interest.

5.2.1.1. Luciferase

[0126] Luciferases are enzymes that emit light in the presence of oxygen and a substrate (luciferin) and which have been used for real-time, low-light imaging of gene expression in cell cultures, individual cells, whole organisms, and transgenic organisms (reviewed by Greer & Szalay, 2002, Luminescence 17(1):43-74).

[0127] As used herein, the term "luciferase" is intended to embrace all luciferases, or recombinant enzymes derived from luciferases which have luciferase activity. The luciferase genes from fireflies have been well characterized, for example, from the *Photinus* and *Luciola* species (see, *e.g.*, International Patent Publication No. WO 95/25798 for *Photinus pyralis*, European Patent Application No. EP 0 524 448 for *Luciola cruciata* and *Luciola lateralis*, and Devine et al., 1993, Biochim. Biophys. Acta 1173(2):121-132 for *Luciola mingrelica)*. Other eucaryotic luciferase genes include, but are not limited to, the click beetle (*Photinus plagiophthalamus*, see, *e.g.*, Wood et al., 1989, Science 244:700-702), the sea panzy (*Renilla reniformis*, see, *e.g.*, Lorenz et al., 1991, Proc Natl Acad Sci U S A 88(10):4438-4442), and the glow worm (*Lampyris noctiluca*, see *e.g.*, Sula-Newby et al., 1996, Biochem J. 313:761-767). The click beetle is unusual in that different members of the species emit bioluminescence of different colors, which emit light at 546 nm (green), 560 nm (yellow-green), 578 nm (yellow) and 593 nm (orange) (see, *e.g*, U.S. Patent Nos. 6,475,719; 6,342,379; and 6,217,847, the disclosures of which are incorporated by reference in their entireties). Bacterial luciferin-luciferase systems include, but are not limited to, the bacterial lux genes of terrestrial *Photorhabdus luminescens* (see, *e.g.*, Manukhov et al., 2000, Genetika 36(3):322-30) and marine bacteria *Vibrio fischeri* and *Vibrio harveyi* (see, *e.g.*, Miyamoto et al., 1988, J Biol Chem. 263(26):13393-9, and Cohn et al., 1983, Proc Natl Acad Sci USA., 80(1):120-3, respectively). The luciferases encompassed by the present

invention also includes the mutant luciferases described in U.S. Patent No. 6,265,177 to Squirrell et al., which is hereby incorporated by reference in its entirety.

[0128] In a preferred embodiment, the luciferase is a firefly luciferase, a renilla luciferase, or a click beetle luciferase, as described in any one of the references listed *supra*, the disclosures of which are incorporated by reference in their entireties.

5.2.1.2. Green Fluorescent Protein

[0129] Green fluorescent protein ("GFP") is a 238 amino acid protein with amino acid residues 65 to 67 involved in the formation of the chromophore which does not require additional substrates or cofactors to fluoresce (see, *e.g.*, Prasher et al., 1992, Gene 111:229-233; Yang et al., 1996, Nature Biotechnol. 14:1252-1256; and Cody et al., 1993, Biochemistry 32:1212-1218).

[0130] As used herein, the term "green fluorescent protein" or "GFP" is intended to embrace all GFPs (including the various forms of GFPs which exhibit colors other than green), or recombinant enzymes derived from GFPs which have GFP activity. In a preferred embodiment, GFP includes green fluorescent protein, yellow fluorescent protein, red fluorescent protein, cyan fluorescent protein, and blue fluorescent protein. The native gene for GFP was cloned from the bioluminescent jellyfish *Aequorea victoria* (see, *e.g.*, Morin et al., 1972, J. Cell Physiol. 77:313-318). Wild type GFP has a major excitation peak at 395 nm and a minor excitation peak at 470 nm. The absorption peak at 470 nm allows the monitoring of GFP levels using standard fluorescein isothiocyanate (FITC) filter sets. Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. For example, mutant GFPs with alanine, glycine, isoleucine, or threonine substituted for serine at position 65 result in mutant GFPs with shifts in excitation maxima and greater fluorescence than wild type protein when excited at 488 nm (see, *e.g.*, Heim et al., 1995, Nature 373:663-664; U.S. Patent No. 5,625,048; Delagrave et al., 1995, Biotechnology 13:151-154; Cormack et al., 1996, Gene 173:33-38; and Cramer et al., 1996, Nature Biotechnol. 14:315-319). The ability to excite GFP at 488 nm permits the use of GFP with standard fluorescence activated cell sorting ("FACS") equipment. In another embodiment, GFPs are isolated from organisms other than the jellyfish, such as, but not limited to, the sea pansy, *Renilla reriformis*.

[0131] Techniques for labeling cells with GFP in general are described in U.S. Patent Nos. 5,491,084 and 5,804,387, which are incorporated by reference in their entireties; Chalfie et al., 1994, Science 263:802-805; Heim et al., 1994, Proc. Natl. Acad. Sci. USA 91:12501-12504; Morise et al., 1974, Biochemistry 13:2656-2662; Ward et al., 1980, Photochem. Photobiol. 31:611-615; Rizzuto et al., 1995, Curr. Biology 5:635-642; and

Kaether & Gerdes, 1995, FEBS Lett 369:267-271. The expression of GFPs in *E. coli* and *C. elegans* are described in U.S. Patent No. 6,251,384 to Tan et al., which is incorporated by reference in its entirety. The expression of GFP in plant cells is discussed in Hu & Cheng, 1995, FEBS Lett 369:331-33, and GFP expression in *Drosophila* is described in Davis et al., 1995, Dev. Biology 170:726-729.

5.2.1.3. Beta-galactosidase

[0132] Beta galactosidase ("b-gal") is an enzyme that catalyzes the hydrolysis of b-galactosides, including lactose, and the galactoside analogs o-nitrophenyl-b-D-galactopyranoside ("ONPG") and chlorophenol red-b-D-galactopyranoside ("CPRG") (see, *e.g.*, Nielsen et al., 1983 Proc Natl Acad Sci USA 80(17):5198-5202; Eustice et al., 1991, Biotechniques 11:739-742; and Henderson et al., 1986, Clin. Chem. 32:1637-1641). The b-gal gene functions well as a reporter gene because the protein product is extremely stable, resistant to proteolytic degradation in cellular lysates, and easily assayed. When ONPG is used as the substrate, b-gal activity can be quantitated with a spectrophotometer or microplate reader.

[0133] As used herein, the term "beta galactosidase" or "b-gal" is intended to embrace all b-gals, including *lacZ* gene products, or recombinant enzymes derived from b-gals which have b-gal activity. The b-gal gene functions well as a reporter gene because the protein product is extremely stable, resistant to proteolytic degradation in cellular lysates, and easily assayed. In an embodiment where ONPG is the substrate, b-gal activity can be quantitated with a spectrophotometer or microplate reader to determine the amount of ONPG converted at 420 nm. In an embodiment when CPRG is the substrate, b-gal activity can be quantitated with a spectrophotometer or microplate reader to determine the amount of CPRG converted at 570 to 595 nm. In yet another embodiment, the b-gal activity can be visually ascertained by plating bacterial cells transformed with a b-gal construct onto plates containing Xgal and IPTG. Bacterial colonies that are dark blue indicate the presence of high b-gal activity and colonies that are varying shades of blue indicate varying levels of b-gal activity.

5.2.1.4. Beta-glucoronidase

[0134] Beta-glucuronidase ("GUS") catalyzes the hydrolysis of a very wide variety of b-glucuronides, and, with much lower efficiency, hydrolyzes some b-galacturonides. GUS is very stable, will tolerate many detergents and widely varying ionic conditions, has no cofactors, nor any ionic requirements, can be assayed at any physiological pH, with an

optimum between 5.0 and 7.8, and is reasonably resistant to thermal inactivation (see, *e.g.*, U.S. Patent No. 5,268,463, which is incorporated by reference in its entirety).

[0135] In one embodiment, the GUS is derived from the *Esherichia coli* b-glucuronidase gene. In alternate embodiments of the invention, the b-glucuronidase encoding nucleic acid is homologous to the *E. coli* b-glucuronidase gene and/or may be derived from another organism or species.

[0136] GUS activity can be assayed either by fluorescence or spectrometry, or any other method described in U.S. Patent No. 5,268,463, the disclosure of which is incorporated by reference in its entirety. For a fluorescent assay, 4-trifluoromethylumbelliferyl b-D-glucuronide is a very sensitive substrate for GUS. The fluorescence maximum is close to 500 nm--bluish green, where very few plant compounds fluoresce or absorb. 4-trifluoromethylumbelliferyl b-D-glucuronide also fluoresces much more strongly near neutral pH, allowing continuous assays to be performed more readily than with MUG. 4-trifluoromethylumbelliferyl b-D-glucuronide can be used as a fluorescent indicator *in vivo*. The spectrophotometric assay is very straightforward and moderately sensitive (Jefferson et al., 1986, Proc. Natl. Acad. Sci. USA 86:8447-8451). A preferred substrate for spectrophotometric measurement is p-nitrophenyl b-D-glucuronide, which when cleaved by GUS releases the chromophore p-nitrophenol. At a pH greater than its pK_a (around 7.15) the ionized chromophore absorbs light at 400-420 nm, giving a yellow color.

5.2.1.5. Beta-lactamase

[0137] Beta-lactamases are nearly optimal enzymes in respect to their almost diffusion-controlled catalysis of b-lactam hydrolysis, making them suited to the task of an intracellular reporter enzyme (see, *e.g.*, Christensen et al., 1990, Biochem. J. 266: 853-861). They cleave the b-lactam ring of b-lactam antibiotics, such as penicillins and cephalosporins, generating new charged moieties in the process (see, *e.g.*, O'Callaghan et al., 1968, Antimicrob. Agents. Chemother. 8: 57-63 and Stratton, 1988, J. Antimicrob. Chemother. 22, Suppl. A: 23-35). A large number of b-lactamases have been isolated and characterized, all of which would be suitable for use in accordance with the present invention (see, *e.g.*, Richmond & Sykes, 1978, Adv.Microb.Physiol. 9:31-88 and Ambler, 1980, Phil. Trans. R. Soc. Lond. [Ser.B.] 289: 321-331, the disclosures of which are incorporated by reference in their entireties).

[0138] The coding region of an exemplary b-lactamase employed has been described in U.S. Patent No. 6,472,205, Kadonaga et al., 1984, J.Biol.Chem. 259: 2149-2154, and Sutcliffe, 1978, Proc. Natl. Acad. Sci. USA 75: 3737-3741, the disclosures of which are incorporated by reference in their entireties. As would be readily apparent to those skilled

in the field, this and other comparable sequences for peptides having b-lactamase activity would be equally suitable for use in accordance with the present invention. The combination of a fluorogenic substrate described in U.S. Patent Nos. 6,472,205, 5,955,604, and 5,741,657, the disclosures of which are incorporated by reference in their entireties, and a suitable b-lactamase can be employed in a wide variety of different assay systems, such as are described in U.S. Patent No. 4,740,459, which is hereby incorporated by reference in its entirety.

5.2.1.6. Chloramphenicol Acetyltransferase

[0139] Chloramphenicol acetyl transferase ("CAT") is commonly used as a reporter gene in mammalian cell systems because mammalian cells do not have detectable levels of CAT activity. The assay for CAT involves incubating cellular extracts with radiolabeled chloramphenicol and appropriate co-factors, separating the starting materials from the product by, for example, thin layer chromatography ("TLC"), followed by scintillation counting (see, *e.g.*, U.S. Patent No. 5,726,041, which is hereby incorporated by reference in its entirety).

[0140] As used herein, the term "chloramphenicol acetyltransferase" or "CAT" is intended to embrace all CATs, or recombinant enzymes derived from CAT which have CAT activity. While it is preferable that a reporter system which does not require cell processing, radioisotopes, and chromatographic separations would be more amenable to high through-put screening, CAT as a reporter gene may be preferable in situations when stability of the reporter gene is important. For example, the CAT reporter protein has an *in vivo* half life of about 50 hours, which is advantageous when an accumulative versus a dynamic change type of result is desired.

5.2.1.7. Secreted Alkaline Phosphatase

[0141] The secreted alkaline phosphatase ("SEAP") enzyme is a truncated form of alkaline phosphatase, in which the cleavage of the transmembrane domain of the protein allows it to be secreted from the cells into the surrounding media. In a preferred embodiment, the alkaline phosphatase is isolated from human placenta.

[0142] As used herein, the term "secreted alkaline phosphatase" or "SEAP" is intended to embrace all SEAP or recombinant enzymes derived from SEAP which have alkaline phosphatase activity. SEAP activity can be detected by a variety of methods including, but not limited to, measurement of catalysis of a fluorescent substrate, immunoprecipitation, HPLC, and radiometric detection. The luminescent method is preferred due to its increased sensitivity over calorimetric detection methods. The advantages of using SEAP is that a

cell lysis step is not required since the SEAP protein is secreted out of the cell, which facilitates the automation of sampling and assay procedures. A cell-based assay using SEAP for use in cell-based assessment of inhibitors of the Hepatitis C virus protease is described in U.S. Patent No. 6,280,940 to Potts et al. which is hereby incorporated by reference in its entirety.

5.2.2. Reporter Gene Constructs

[0143] The invention provides reporter gene constructs for use in the reporter gene-based assays described herein for the identification of compounds that modulate untranslated region-dependent expression of a target gene. The reporter gene constructs of the invention comprise one or more reporter genes fused to one or more untranslated regions. For example, specific RNA sequences, RNA structural motifs, and/or RNA structural elements that are known or suspected to modulate untranslated region-dependent expression of a target gene may be fused to the reporter gene.

[0144] The present invention provides for a reporter gene flanked by one or more untranslated regions (*e.g.*, the 5' UTR, 3' UTR, or both the 5' UTR and 3' UTR of the target gene). The present invention also provides for a reporter gene flanked by one or more UTRs of a target gene, said UTRs containing one or more mutations (*e.g.*, one or more substitutions, deletions and/or additions). In a preferred embodiment, the reporter gene is flanked by both 5' and 3' UTRs so that compounds that interfere with an interaction between the 5' and 3' UTRs can be identified. In another preferred embodiment, a stable hairpin secondary structure is inserted into the UTR, preferably the 5' UTR of the target gene. For example, in cases where the 5' UTR possesses IRES activity, the addition of a stable hairpin secondary structure in the 5' UTR can be used to separate cap-dependent from cap-independent translation (see, *e.g.*, Muhlrad et al., 1995, Mol. Cell. Biol. 15(4):2145-56, the disclosure of which is incorporated by reference in its entirety). In another embodiment, an intron is inserted into a UTR (preferably, the 5' UTR) or at the 5' end of an ORF of a target gene. For example, but not by limitation, in cases where an RNA possesses instability elements, an intron, *e.g.*, the human elongation factor one alpha (EF-1 alpha) first intron, can be cloned into a UTR (preferably, the 5' UTR) or a 5' end of the ORF to increase expression (see, *e.g.*, Kim et al., 2002, J Biotechnol 93(2):183-7, the disclosure of which is incorporated by reference in its entirety). In a preferred embodiment, both a stable hairpin secondary structure and an intron are added to the reporter gene construct. In a more preferred embodiment, the stable hairpin secondary structure is cloned into the 5' UTR and the intron is added at the 5' end of the ORF of the reporter gene.

[0145] The reporter gene can be positioned such that the translation of that reporter gene is dependent upon the mode of translation initiation, such as, but not limited to, cap-dependent translation or cap-independent translation (*i.e.*, translation via an internal ribosome entry site). Alternatively, where the UTR contains an upstream open reading frame, the reporter gene can be positioned such that the reporter protein is translated only in the presence of a compound that shifts the reading frame of the UTR so that the formerly untranslated open reading frame is then translated.

[0146] The reporter gene constructs can be monocistronic or multicistronic. A multicistronic reporter gene construct may encode 2, 3, 4, 5, 6, 7, 8, 9, 10 or more, or in the range of 2-5, 5-10 or 10-20 reporter genes. For example, a dicistronic reporter gene construct comprising in the following order a promoter, a first reporter gene, a 5' UTR of a target gene, a second reporter gene and optionally, a 3' UTR of a target gene. In such a reporter construct, the transcription of both reporter genes is driven by the promoter, whereas the translation of the mRNA from the first reporter gene is by a cap-dependent scanning mechanism and the translation of the mRNA from the second reporter gene is by a cap-independent mechanism by an IRES. The IRES-dependent translation of the mRNA of the second reporter gene can be normalized against cap-dependent translation.

5.2.3. Expression of Reporter Gene Constructs in Cells

5.2.3.1. Vectors

[0147] The nucleotide sequence coding for a reporter gene can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the target gene or the reporter gene. A variety of host-vector systems may be utilized to express the reporter gene. These include, but are not limited to, mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA; and stable cell lines generated by transformation using a selectable marker. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, the reporter gene is expressed, or a fusion protein comprising the reporter gene and ORF of a fragment thereof, of the target gene is expressed.

[0148] Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric nucleic acid consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of the reporter gene construct may be regulated by a second nucleic acid sequence so that the reporter gene is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a reporter gene construct may be controlled by any promoter/enhancer element known in the art, such as a constitutive promoter, a tissue-specific promoter, or an inducible promoter. Specific examples of promoters which may be used to control gene expression include, but are not limited to, the SV40 early promoter region (Bernoist & Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver

(Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

[0149] In a specific embodiment, a vector is used that comprises a promoter operably linked to a reporter gene flanked by one or more UTRs of a target gene, origins of replication from one or more species, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene). In a preferred embodiment, the vectors are CMV vectors, T7 vectors, lac vectors, pCEP4 vectors, or 5.0/FRT vectors.

[0150] In a specific embodiment, an expression construct is made by amplifying the 5' and/or 3' UTRs of a target gene and ligating the UTRs to a reporter gene such as luciferase, and subcloning them into a pT-Adv vector (Clontech Laboratories, Palo Alto, California). It is understood by one of skill in the art that the construction of the reporter plasmid may require the construction of intermediate plasmids if several ligations are involved.

[0151] Expression vectors containing the reporter gene construct of the present invention can be identified by four general approaches: (a) nucleic acid sequencing, (b) nucleic acid hybridization, (c) presence or absence of "marker" nucleic acid functions, and (d) expression of inserted sequences. In the first approach, the presence of the UTRs and/or the reporter gene inserted in an expression vector can be detected by sequencing. In the second approach, the presence of the UTRs and/or the reporter gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to the inserted UTRs and/or reporter gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" nucleic acid functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, *etc.*) caused by the insertion of the nucleic acid of interest, *i.e.*, the reporter gene construct, in the vector. For example, if the nucleic acid of interest is inserted within the marker nucleic acid sequence of the vector, recombinants containing the insert can be identified by the absence of the marker nucleic acid function. In the fourth approach,

recombinant expression vectors can be identified by assaying the reporter gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the particular reporter gene.

[0152] In a preferred embodiment, the reporter gene constructs are cloned into stable cell line expression vectors. In a preferred embodiment, the stable cell line expression vector contains a site specific genomic integration site, such as but not limited to, pCMP1 (see, *e.g.*, FIG. 8C in Example 10). In another preferred embodiment, the reporter gene construct is cloned into an episomal mammalian expression vector, such as, but not limited to, pCMR2 (see, *e.g.*, FIG. 8B in Example 10).

5.2.3.2. Transfection

[0153] Once a vector encoding the appropriate gene has been synthesized, a host cell is transformed or transfected with the vector of interest. The use of stable transformants is preferred. In a preferred embodiment, the host cell is a mammalian cell. In a more preferred embodiment, the host cell is a human cell. In another embodiment, the host cells are primary cells isolated from a tissue or other biological sample of interest. Host cells that can be used in the methods of the present invention include, but are not limited to, hybridomas, pre-B cells, 293 cells, 293T cells, HeLa cells, HepG2 cells, K562 cells, 3T3 cells, MCF7 cells, SkBr3 cells, or BT474 cells. In another preferred embodiment, the host cells are derived from tissue specific to the target gene. In yet another preferred embodiment, the host cells are immortalized cell lines derived from a source, *e.g.*, a tissue, specific to the target gene. Other host cells that can be used in the present invention include, but are not limited to, bacterial cells, yeast cells, virally-infected cells, or plant cells.

[0154] Transformation may be by any known method for introducing polynucleotides into a host cell, for example by packaging the polynucleotide in a virus and transducing a host cell with the virus, and by direct uptake of the polynucleotide. The transformation procedure used depends upon the host to be transformed. Bacterial transformation by direct uptake generally employs treatment with calcium or rubidium chloride (see, *e.g.*, Cohen, 1972, Proc. Nat. Acad. Sci. USA 69:2110 and Maniatis et al., 1982, "Molecular Cloning; A Laboratory Manual" (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.). Yeast transformation by direct uptake may be carried out using the method of Schiestl & Gietz, 1989, Current Genetics 16:339-346 or Hinnen et al., 1978, Proc. Nat. Acad. Sci. USA 75:1929. Mammalian transformations (*i.e.*, transfections) by direct uptake may be conducted using the calcium phosphate precipitation method of Graham & Van der Eb, 1978, Virol. 52:546, or the various known modifications thereof. Other methods for

introducing recombinant polynucleotides into cells, particularly into mammalian cells, include dextran-mediated transfection, calcium phosphate mediated transfection, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the polynucleotides into nuclei. Such methods are well-known to one of skill in the art.

[0155] In a preferred embodiment, stable cell lines containing the constructs of interest are generated for high throughput screening. Such stable cells lines may be generated by introducing a reporter gene construct comprising a selectable marker, allowing the cells to grow for 1-2 days in an enriched medium, and then growing the cells on a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

[0156] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (see, *e.g.,* Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (see, *e.g.,* Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (see, *e.g.,* Lowy et al., 1980, Cell 22:817) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (see, *e.g.,* Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567 and O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (see, *e.g.,* Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 gene (see, *e.g.*, Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin genes (see, *e.g.,* Santerre et al., 1984, Gene 30:147).

5.2.4. Cell-Free Extracts

[0157] The invention provides for the translation of the reporter gene constructs in a cell-free system. Techniques for practicing this specific aspect of this invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, and recombinant DNA manipulation and production, which are routinely practiced by one of skill in the art. See, *e.g.*, Sambrook, 1989, Molecular Cloning, A Laboratory Manual, Second Edition; DNA Cloning, Volumes I and II (Glover, Ed. 1985); and Transcription and Translation (Hames & Higgins, Eds. 1984).

[0158] Any technique well-known to one of skill in the art may be used to generate cell-free extracts for translation *in vitro* (otherwise referred to herein as cell-free translation mixtures). For example, the cell-free extracts for *in vitro* translation reactions can be

generated by centrifuging cells and clarifying the supernatant. The cell extracts for the present invention is about a S1 (*i.e.*, the supernatant from a 1,000 x g spin) to about a S500 extract (*i.e.*, the supernatant from a 500,000 x g spin), preferably about a S10 (*i.e.*, the supernatant from a 10,000 x g spin) to S250 (*i.e.*, the supernatant from a 250,000 x g spin) extract. In some embodiments, about a S50 (*i.e.*, the supernatant from a 50,000 x g spin) to S100 (*i.e.*, the supernatant from a 100,000 x g spin) extract is preferred.

[0159] The cell-free translation extract may be isolated from cells of any species origin. For example, the cell-free translation extract may be isolated from human cells (*e.g.*, HeLA cells), 293 cells, Vero cells, yeast, mouse cells (*e.g.*, cultured mouse cells), rat cells (*e.g.*, cultured rat cells), Chinese hamster ovary (CHO) cells, Xenopus oocytes, rabbit reticulocytes, primary cells, cancer cells (*e.g.*, undifferentiated cancer cells), cell lines, wheat germ, rye embryo, or bacterial cell extract (see, *e.g.*, Krieg & Melton, 1984, Nature 308:203 and Dignam et al., 1990 Methods Enzymol. 182:194-203). Alternatively, the cell-free translation extract, *e.g.*, rabbit reticulocyte lysates and wheat germ extract, can be purchased from, *e.g.*, Promega, (Madison, WI). It is preferred that the cells from which the cell-free extract is obtained do not endogenously express a target gene of interest. In a preferred embodiment, the cell-free extract is an extract isolated from human cells. In a more preferred embodiment, the human cells are HeLa cells.

5.3. Libraries of Compounds

[0160] Libraries screened using the methods of the present invention can comprise a variety of types of compounds. Examples of libraries that can be screened in accordance with the methods of the invention include, but are not limited to, peptoids; random biooligomers; diversomers such as hydantoins, benzodiazepines and dipeptides; vinylogous polypeptides; nonpeptidal peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; carbohydrate libraries; and small molecule libraries (preferably small organic molecules). In some embodiments, the compounds in the libraries screened are nucleic acid or peptide molecules. In a non-limiting example, peptide molecules can exist in a phage display library. In other embodiments, the types of compounds include, but are not limited to, peptide analogs including peptides comprising non-naturally occurring amino acids, *e.g.*, D-amino acids, phosphorous analogs of amino acids, such as α-amino phosphoric acids and α-amino phosphoric acids, or amino acids having non-peptide linkages, nucleic acid analogs such as phosphorothioates and PNAs, hormones, antigens, synthetic or naturally occurring drugs, opiates, dopamine, serotonin, catecholamines, thrombin, acetylcholine, prostaglandins, organic molecules, pheromones,

adenosine, sucrose, glucose, lactose and galactose. Libraries of polypeptides or proteins can also be used in the assays of the invention.

[0161] In a preferred embodiment, the combinatorial libraries are small organic molecule libraries including, but not limited to, benzodiazepines, isoprenoids, beta carbalines, thiazolidinones, metathiazanones, pyrrolidines, morpholino compounds, and benzodiazepines. In another embodiment, the combinatorial libraries comprise peptoids; random bio-oligomers; benzodiazepines; diversomers such as hydantoins, benzodiazepines and dipeptides, vinylogous polypeptides; nonpeptidal peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; or carbohydrate libraries. Combinatorial libraries are themselves commercially available (see, *e.g.*, ComGenex, Princeton, New Jersey; Asinex, Moscow, Ru, Tripos, Inc., St. Louis, Missouri; ChemStar, Ltd, Moscow, Russia; 3D Pharmaceuticals, Exton, Pennsylvania; Martek Biosciences, Columbia, Maryland; *etc.*).

[0162] In a preferred embodiment, the library is preselected so that the compounds of the library are more amenable for cellular uptake. For example, compounds are selected based on specific parameters such as, but not limited to, size, lipophilicity, hydrophilicity, and hydrogen bonding, which enhance the likelihood of compounds getting into the cells. In another embodiment, the compounds are analyzed by three-dimensional or four-dimensional computer computation programs.

[0163] The combinatorial compound library for use in accordance with the methods of the present invention may be synthesized. There is a great interest in synthetic methods directed toward the creation of large collections of small organic compounds, or libraries, which could be screened for pharmacological, biological or other activity. The synthetic methods applied to create vast combinatorial libraries are performed in solution or in the solid phase, *i.e.*, on a solid support. Solid-phase synthesis makes it easier to conduct multi-step reactions and to drive reactions to completion with high yields because excess reagents can be easily added and washed away after each reaction step. Solid-phase combinatorial synthesis also tends to improve isolation, purification and screening. However, the more traditional solution phase chemistry supports a wider variety of organic reactions than solid-phase chemistry.

[0164] Combinatorial compound libraries of the present invention may be synthesized using the apparatus described in U.S. Patent No. 6,190,619 to Kilcoin et al., which is hereby incorporated by reference in its entirety. U.S. Patent No. 6,190,619 discloses a synthesis apparatus capable of holding a plurality of reaction vessels for parallel synthesis of multiple discrete compounds or for combinatorial libraries of compounds.

[0165] In one embodiment, the combinatorial compound library can be synthesized in solution. The method disclosed in U.S. Patent No. 6,194,612 to Boger et al., which is hereby incorporated by reference in its entirety, features compounds useful as templates for solution phase synthesis of combinatorial libraries. The template is designed to permit reaction products to be easily purified from unreacted reactants using liquid/liquid or solid/liquid extractions. The compounds produced by combinatorial synthesis using the template will preferably be small organic molecules. Some compounds in the library may mimic the effects of non-peptides or peptides. In contrast to solid phase synthesize of combinatorial compound libraries, liquid phase synthesis does not require the use of specialized protocols for monitoring the individual steps of a multistep solid phase synthesis (Egner et al., 1995, J.Org. Chem. 60:2652; Anderson et al., 1995, J. Org. Chem. 60:2650; Fitch et al., 1994, J. Org. Chem. 59:7955; Look et al., 1994, J. Org. Chem. 49:7588; Metzger et al., 1993, Angew. Chem., Int. Ed. Engl. 32:894; Youngquist et al., 1994, Rapid Commun. Mass Spect. 8:77; Chu et al., 1995, J. Am. Chem. Soc. 117:5419; Brummel et al., 1994, Science 264:399; and Stevanovic et al., 1993, Bioorg. Med. Chem. Lett. 3:431).

[0166] Combinatorial compound libraries useful for the methods of the present invention can be synthesized on solid supports. In one embodiment, a split synthesis method, a protocol of separating and mixing solid supports during the synthesis, is used to synthesize a library of compounds on solid supports (see *e.g.*, Lam et al., 1997, Chem. Rev. 97:41-448; Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926 and references cited therein). Each solid support in the final library has substantially one type of compound attached to its surface. Other methods for synthesizing combinatorial libraries on solid supports, wherein one product is attached to each support, will be known to those of skill in the art (*see, e.g.*, Nefzi et al., 1997, Chem. Rev. 97:449-472).

[0167] As used herein, the term "solid support" is not limited to a specific type of solid support. Rather a large number of supports are available and are known to one skilled in the art. Solid supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, polystyrene beads, alumina gels, and polysaccharides. A suitable solid support may be selected on the basis of desired end use and suitability for various synthetic protocols. For example, for peptide synthesis, a solid support can be a resin such as p-methylbenzhydrylamine (pMBHA) resin (Peptides International, Louisville, KY), polystyrenes (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, *etc.*), including chloromethylpolystyrene, hydroxymethylpolystyrene and aminomethylpolystyrene, poly (dimethylacrylamide)-grafted styrene co-divinyl-benzene (*e.g.*, POLYHIPE resin, obtained from Aminotech, Canada), polyamide resin (obtained

from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (*e.g.*, TENTAGEL or ARGOGEL, Bayer, Tubingen, Germany) polydimethylacrylamide resin (obtained from Milligen/Biosearch, California), or Sepharose (Pharmacia, Sweden).

[0168] In some embodiments of the present invention, compounds can be attached to solid supports via linkers. Linkers can be integral and part of the solid support, or they may be nonintegral that are either synthesized on the solid support or attached thereto after synthesis. Linkers are useful not only for providing points of compound attachment to the solid support, but also for allowing different groups of molecules to be cleaved from the solid support under different conditions, depending on the nature of the linker. For example, linkers can be, *inter alia*, electrophilically cleaved, nucleophilically cleaved, photocleavable, enzymatically cleaved, cleaved by metals, cleaved under reductive conditions or cleaved under oxidative conditions. In a preferred embodiment, the compounds are cleaved from the solid support prior to high throughput screening of the compounds.

5.4. Reporter Gene-Based Screening Assays

5.4.1. Cell-Based Assays

[0169] After a vector containing the reporter gene construct is transformed or transfected into a host cell and a compound library is synthesized or purchased or both, the cells are used to screen the library to identify compounds that modulate untranslated region-dependent expression of a target gene. In a preferred embodiment, the cells are stably transfected with the reporter gene construct. The reporter gene-based assays may be conducted by contacting a compound or a member of a library of compounds with a cell genetically engineered to express a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions of a target gene, and measuring the expression of said reporter gene. The alteration in reporter gene expression relative to a previously determined reference range, the absence of the compound or a control in such reporter-gene based assays indicates that a particular compound modulates untranslated region-dependent expression of a target gene. In a preferred embodiment, a negative control (*e.g.*, PBS or another agent that is known to have no effect on the expression of the reporter gene) and a positive control (*e.g.*, an agent that is known to have an effect on the expression of the reporter gene, preferably an agent that effects untranslated region-dependent expression) are included in the cell-based assays described herein.

[0170] The step of contacting a compound or a member of a library of compounds with a cell genetically engineered to express a reporter gene operably linked to one or more

untranslated regions may be conducted under physiologic conditions. In specific embodiment, a compound or a member of a library of compounds is added to the cells in the presence of an aqueous solution. In accordance with this embodiment, the aqueous solution may comprise a buffer and a combination of salts, preferably approximating or mimicking physiologic conditions. Alternatively, the aqueous solution may comprise a buffer, a combination of salts, and a detergent or a surfactant. Examples of salts which may be used in the aqueous solution include, but not limited to, KCl, NaCl, and/or $MgCl_2$. The optimal concentration of each salt used in the aqueous solution is dependent on the cells and compounds used and can be determined using routine experimentation.

[0171] The invention provides for contacting a compound or a member of a library of compounds with a cell genetically engineering to express a reporter gene operably linked to one or more untranslated regions for a specific period of time. For example, the contacting can take place for about 1 minute, 2 minutes, 3 minutes, 4, minutes, 5, minutes, 10 minutes, 15 minutes, 20 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 10 hours, 15 hours, 20 hours, 1 day, 2 days, 3 days, 4 days, 5 days, or 1 week. In a preferred embodiment, the contacting is about 15 hours, *i.e.*, overnight. The contacting can take place for about 1 minute to 1 week, preferably about 5 minutes to 5 days, more preferably about 10 minutes to 2 days, and even more preferably about 1 hour to 1 day.

[0172] In one embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) expressing a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions of said target gene in a cell; (b) contacting said cell with a member of a library of compounds; and (c) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent regulation of expression is identified if the expression of said reporter gene in the presence of a compound is altered relative to a previously determined reference range or the expression of said reporter gene in the absence of said compound or the presence of a control (*e.g.*, phosphate buffered saline ("PBS")). In another embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) contacting a member of a library of compounds with a cell containing a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions of said target gene; and (b) detecting a reporter protein translated from said reporter gene, wherein detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression is identified if the expression of said reporter gene in the presence of a compound is altered relative to a

previously determined reference range or the expression of said reporter gene in the absence of said compound or the presence of a control (*e.g.*, PBS).

[0173] The invention also provides methods of identifying compounds that upregulate or down-regulate untranslated region-dependent expression of a target gene utlitizing the cell-based reporter gene assays described herein. In a specific embodiment, the invention provides a method of upregulating untranslated region-dependent expression of a target gene, said method comprising (a) contacting a compound with a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that upregulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of a compound is increased relative a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS). In another embodiment, the invention provides a method of down-regulating untranslated region-dependent expression of a target gene, said method comprising (a) contacting a compound with a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that down-regulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of a compound is decreased relative a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS).

[0174] The present invention provides methods of identifying environmental stimuli (*e.g.*, exposure to different concentrations of CO_2 and/or O_2,stress and different pHs) that modulate untranslated region-dependent expression of a target gene utilizing the cell-based reporter gene assays described herein. In particular, the invention provides a method of identifying an environmental stimulus, said method comprising (a) contacting a cell containing a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene with an environmental stimulus; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that modulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of an environmental stimuli is altered relative to a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS). In a specific embodiment, the environmental stimuli

is not hypoxia. In another embodiment, the environmental stimuli does not include a compound.

[0175] The expression of a reporter gene in the cell-based reporter-gene assays may be detected by any technique well-known to one of skill in the art. Methods for detecting the expression of a reporter gene will vary with the reporter gene used. Assays for the various reporter genes are well-known to one of skill in the art. For example, as described in Section 5.2.1., luciferase, beta-galactosidase ("b-gal"), beta-glucoronidase ("GUS"), beta-lactamase, chloramphenicol acetyltransferase ("CAT"), and alkaline phosphatase ("AP") are enzymes that can be analyzed in the presence of a substrate and could be amenable to high throughput screening. For example, the reaction products of luciferase, beta-galactosidase ("b-gal"), and alkaline phosphatase ("AP") are assayed by changes in light imaging (*e.g.*, luciferase), spectrophotometric absorbance (*e.g.*, b-gal), or fluorescence (*e.g.*, AP). Assays for changes in light output, absorbance, and/or fluorescence are easily adapted for high throughput screening. For example, b-gal activity can be measured with a microplate reader. Green fluorescent protein ("GFP") activity can be measured by changes in fluorescence. For example, in the case of mutant GFPs that fluoresce at 488 nm, standard fluorescence activated cell sorting ("FACS") equipment can be used to separate cells based upon GFP activity.

[0176] Alterations in the expression of a reporter gene may be determined by comparing the level of expression of the reporter gene to a negative control (*e.g.*, PBS or another agent that is known to have no effect on the expression of the reporter gene) and optionally, a positive control (*e.g.*, an agent that is known to have an effect on the expression of the reporter gene, preferably an agent that effects untranslated region-dependent expression). Alternatively, alterations in the expression of a reporter gene may be determined by comparing the level of expression of the reporter gene to a previously determined reference range.

5.4.2. Cell-Free Assays

[0177] After a vector containing the reporter gene construct is produced, a cell-free translation extract is generated or purchased, and a compound library is synthesized or purchased or both, the cell-free translation extract and nucleic acid are used to screen the library to identify compounds that modulate untranslated region-dependent expression of a target gene. The reporter gene-based assays may be conducted in a cell-free manner by contacting a compound or a member of a library of compounds with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions of a target gene, and measuring the expression of said reporter gene.

The alteration in reporter gene expression relative to a previously determined reference range, the absence of a compound or a control in such reporter-gene based assays indicates that a particular compound modulates untranslated region-dependent expression of a target gene. In a preferred embodiment, a negative control (*e.g.*, PBS or another agent that is known to have no effect on the expression of the reporter gene) and a positive control (*e.g.*, an agent that is known to have an effect on the expression of the reporter gene, preferably an agent that effects untranslated region-dependent expression) are included in the cell-free assays described herein.

[0178] The step of contacting a compound or a member of a library of compounds with a cell-free translation mixture containing a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions may be conducted under conditions approximating or mimicking physiologic conditions. In specific embodiment, a compound or a member of a library of compounds is added to the cells in the presence of an aqueous solution. In accordance with this embodiment, the aqueous solution may comprise a buffer and a combination of salts, preferably approximating or mimicking physiologic conditions. Alternatively, the aqueous solution may comprise a buffer, a combination of salts, and a detergent or a surfactant. Examples of salts which may be used in the aqueous solution include, but not limited to, KCl, NaCl, and/or $MgCl_2$. The optimal concentration of each salt used in the aqueous solution is dependent on the cells and compounds used and can be determined using routine experimentation.

[0179] The invention provides for contacting a compound or a member of a library of compounds with a cell genetically engineering to express a reporter gene operably linked to one or more untranslated regions for a specific period of time. For example, the contacting can take place for about 1 minute, 2 minutes, 3 minutes, 4, minutes, 5, minutes, 10 minutes, 15 minutes, 20 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 10 hours, 15 hours, 20 hours, 1 day, 2 days, 3 days, 4 days, 5 days, or 1 week. In a preferred embodiment, the contacting is about 15 hours, *i.e.*, overnight. The contacting can take place for about 1 minute to 1 week, preferably about 5 minutes to 5 days, more preferably about 10 minutes to 2 days, and even more preferably about 1 hour to 1 day.

[0180] In a specific embodiment, the invention provides a method for identifying a compound that modulates untranslated region-dependent expression of a target gene, said method comprising: (a) contacting a member of a library of compounds with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one or more untranslated regions of said target gene; and (b) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent

expression is identified if the expression of said reporter gene in the presence of a compound is altered relative to a previously determined reference range or the expression of said reporter gene in the absence of said compound or the presence of a control (*e.g.*, PBS).

[0181] The invention also provides methods of identifying compounds that upregulate or down-regulate untranslated region-dependent expression of a target gene utlitizing the cell-free reporter gene assays described herein. In a specific embodiment, the invention provides a method of upregulating untranslated region-dependent expression of a target gene, said method comprising (a) contacting a compound with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that upregulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of a compound is increased relative a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS). In another embodiment, the invention provides a method of down-regulating untranslated region-dependent expression of a target gene, said method comprising (a) contacting a compound with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to one, two, three or more untranslated regions of said target gene; and (b) detecting a reporter gene protein translated from said reporter gene, wherein a compound that down-regulates untranslated region dependent expression is identified if the expression of said reporter gene in the presence of a compound is decreased relative to a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS).

[0182] The activity of a compound in the *in vitro* translation mixture can be determined by assaying the activity of a reporter protein encoded by a reporter gene, or alternatively, by quantifying the expression of the reporter gene by, for example, labeling the *in vitro* translated protein (*e.g.*, with ^{35}S-labeled methionine), northern blot analysis, RT-PCR or by immunological methods, such as western blot analysis or immunoprecipitation. Such methods are well-known to one of skill in the art.

5.4.3. Direct Binding Assays

[0183] Compounds that modulate untranslated region-dependent expression of a target gene can be identified by direct binding assays. In this embodiment, the target RNA comprises one or more untranslated regions, and preferably contains at least one element of an untranslated region. Such assays are described in International Patent Publication Nos. WO 02/083837 and WO 02/083953, the disclosures of which are hereby incorporated by

reference in their entireties. Briefly, direct binding assays may be conducted by attaching a library of compounds to solid supports, *e.g.*, polymer beads, with each solid support having substantially one type of compound attached to its surface. The plurality of solid supports of the library is exposed in aqueous solution to target RNA having a detectable label, forming a dye-labeled target RNA:support-attached compound complex. Binding of a target RNA molecule to a particular compound labels the solid support, *e.g.*, bead, comprising the compound, which can be physically separated from other, unlabeled solid supports. Once labeled solid supports are identified, the chemical structures of the compounds thereon can be determined by, *e.g.*, by reading a code on the solid support that correlates with the structure of the attached compound.

[0184] Alternatively, direct binding assays may be conducted by contacting a target RNA having a detectable label with a member of a library of compounds free in solution, in labeled tubes or microtiter wells, or a microarray. Compounds in the library that bind to the labeled target RNA will form a detectably labeled complex that can be identified and removed from the uncomplexed, unlabeled compounds in the library, and from uncomplexed, labeled target RNA, by a variety of methods including, but not limited to, methods that differentiate changes in the electrophoretic, chromatographic, or thermostable properties of the complexed target RNA.

5.4.3.1. Electrophoresis

[0185] Methods for separation of the complex of a target RNA bound to a compound from the unbound RNA comprises any method of electrophoretic separation, including but not limited to, denaturing and non-denaturing polyacrylamide gel electrophoresis, urea gel electrophoresis, gel filtration, pulsed field gel electrophoresis, two dimensional gel electrophoresis, continuous flow electrophoresis, zone electrophoresis, agarose gel electrophoresis, and capillary electrophoresis.

[0186] In a preferred embodiment, an automated electrophoretic system comprising a capillary cartridge having a plurality of capillary tubes is used for high-throughput screening of compounds bound to target RNA. Such an apparatus for performing automated capillary gel electrophoresis is disclosed in U.S. Patent Nos. 5,885,430; 5,916,428; 6,027,627; and 6,063,251, the disclosures of which are incorporated by reference in their entireties.

[0187] The device disclosed in U.S. Patent No. 5,885,430, which is incorporated by reference in its entirety, allows one to simultaneously introduce samples into a plurality of capillary tubes directly from microtiter trays having a standard size. U.S. Patent No. 5,885,430 discloses a disposable capillary cartridge which can be cleaned between

electrophoresis runs, the cartridge having a plurality of capillary tubes. A first end of each capillary tube is retained in a mounting plate, the first ends collectively forming an array in the mounting plate. The spacing between the first ends corresponds to the spacing between the centers of the wells of a microtiter tray having a standard size. Thus, the first ends of the capillary tubes can simultaneously be dipped into the samples present in the tray's wells. The cartridge is provided with a second mounting plate in which the second ends of the capillary tubes are retained. The second ends of the capillary tubes are arranged in an array which corresponds to the wells in the microtiter tray, which allows for each capillary tube to be isolated from its neighbors and therefore free from cross-contamination, as each end is dipped into an individual well.

[0188] Plate holes may be provided in each mounting plate and the capillary tubes inserted through these plate holes. In such a case, the plate holes are sealed airtight so that the side of the mounting plate having the exposed capillary ends can be pressurized. Application of a positive pressure in the vicinity of the capillary openings in this mounting plate allows for the introduction of air and fluids during electrophoretic operations and also can be used to force out gel and other materials from the capillary tubes during reconditioning. The capillary tubes may be protected from damage using a needle comprising a cannula and/or plastic tubes, and the like when they are placed in these plate holes. When metallic cannula or the like are used, they can serve as electrical contacts for current flow during electrophoresis. In the presence of a second mounting plate, the second mounting plate is provided with plate holes through which the second ends of the capillary tubes project. In this instance, the second mounting plate serves as a pressure containment member of a pressure cell and the second ends of the capillary tubes communicate with an internal cavity of the pressure cell. The pressure cell is also formed with an inlet and an outlet. Gels, buffer solutions, cleaning agents, and the like may be introduced into the internal cavity through the inlet, and each of these can simultaneously enter the second ends of the capillaries.

[0189] In another preferred embodiment, the automated electrophoretic system can comprise a chip system consisting of complex designs of interconnected channels that perform and analyze enzyme reactions using part of a channel design as a tiny, continuously operating electrophoresis material, where reactions with one sample are going on in one area of the chip while electrophoretic separation of the products of another sample is taking place in a different part of the chip. Such a system is disclosed in U.S. Patent Nos. 5,699,157; 5,842,787; 5,869,004; 5,876,675; 5,942,443; 5,948,227; 6,042,709; 6,042,710; 6,046,056; 6,048,498; 6,086,740; 6,132,685; 6,150,119; 6,150,180; 6,153,073; 6,167,910;

6,171,850; and 6,186,660, the disclosures of which are incorporated by reference in their entireties.

[0190] The system disclosed in U.S. Patent No. 5,699,157, which is hereby incorporated by reference in its entirety, provides for a microfluidic system for high-speed electrophoretic analysis of subject materials for applications in the fields of chemistry, biochemistry, biotechnology, molecular biology and numerous other areas. The system has a channel in a substrate, a light source and a photoreceptor. The channel holds subject materials in solution in an electric field so that the materials move through the channel and separate into bands according to species. The light source excites fluorescent light in the species bands and the photoreceptor is arranged to receive the fluorescent light from the bands. The system further has a means for masking the channel so that the photoreceptor can receive the fluorescent light only at periodically spaced regions along the channel. The system also has an unit connected to analyze the modulation frequencies of light intensity received by the photoreceptor so that velocities of the bands along the channel are determined, which allows the materials to be analyzed.

[0191] The system disclosed in U.S. Patent No. 5,699,157 also provides for a method of performing high-speed electrophoretic analysis of subject materials, which comprises the steps of holding the subject materials in solution in a channel of a microfluidic system; subjecting the materials to an electric field so that the subject materials move through the channel and separate into species bands; directing light toward the channel; receiving light from periodically spaced regions along the channel simultaneously; and analyzing the frequencies of light intensity of the received light so that velocities of the bands along the channel can be determined for analysis of said materials. The determination of the velocity of a species band determines the electrophoretic mobility of the species and its identification.

[0192] U.S. Patent No. 5,842,787, which is hereby incorporated by reference in its entirety, is generally directed to devices and systems employ channels having, at least in part, depths that are varied over those which have been previously described (such as the device disclosed in U.S. Patent No. 5,699,157), wherein said channel depths provide numerous beneficial and unexpected results such as but not limited to, a reduction in sample perturbation, reduced non-specific sample mixture by diffusion, and increased resolution.

[0193] In another embodiment, the electrophoretic method of separation comprises polyacrylamide gel electrophoresis. In a preferred embodiment, the polyacrylamide gel electrophoresis is non-denaturing, so as to differentiate the mobilities of the target RNA bound to a compound from free target RNA. If the polyacrylamide gel electrophoresis is

denaturing, then the target RNA:compound complex must be cross-linked prior to electrophoresis to prevent the disassociation of the target RNA from the compound during electrophoresis. Such techniques are well known to one of skill in the art.

[0194] In one embodiment of the method, the binding of compounds to target nucleic acid can be detected, preferably in an automated fashion, by gel electrophoretic analysis of interference footprinting. RNA can be degraded at specific base sites by enzymatic methods such as ribonucleases A, U_2, CL_3, T_1, Phy M, and *B. cereus* or chemical methods such as diethylpyrocarbonate, sodium hydroxide, hydrazine, piperidine formate, dimethyl sulfate, [2,12-dimethyl-3,7,11,17-tetraazacyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaenato] nickel(II) (NiCR), cobalt(II)chloride, or iron(II) ethylenediaminetetraacetate (Fe-EDTA) as described for example in Zheng et al., 1999, Biochem. 37:2207-2214; Latham & Cech, 1989, Science 245:276-282; and Sambrook et al., 2001, in Molecular Cloning: A Laboratory Manual, pp 12.61-12.73, Cold Spring Harbor Laboratory Press, and the references cited therein, which are hereby incorporated by reference in their entireties. The specific pattern of cleavage sites is determined by the accessibility of particular bases to the reagent employed to initiate cleavage and, as such, is therefore is determined by the three-dimensional structure of the RNA.

[0195] The interaction of small molecules with a target nucleic acid can change the accessibility of bases to these cleavage reagents both by causing conformational changes in the target nucleic acid or by covering a base at the binding interface. When a compound binds to the nucleic acid and changes the accessibility of bases to cleavage reagents, the observed cleavage pattern will change. This method can be used to identify and characterize the binding of small molecules to RNA as described, for example, by Prudent et al., 1995, J. Am. Chem. Soc. 117:10145-10146 and Mei et al., 1998, Biochem. 37:14204-14212.

[0196] In the preferred embodiment of this technique, the detectably labeled target nucleic acid is incubated with an individual compound and then subjected to treatment with a cleavage reagent, either enzymatic or chemical. The reaction mixture can be preferably be examined directly, or treated further to isolate and concentrate the nucleic acid. The fragments produced are separated by electrophoresis and the pattern of cleavage can be compared to a cleavage reaction performed in the absence of compound. A change in the cleavage pattern directly indicates that the compound binds to the target nucleic acid. Multiple compounds can be examined both in parallel and serially.

[0197] Other embodiments of electrophoretic separation include, but are not limited to urea gel electrophoresis, gel filtration, pulsed field gel electrophoresis, two dimensional gel

electrophoresis, continuous flow electrophoresis, zone electrophoresis, and agarose gel electrophoresis.

5.4.3.2. Size Exclusion Chromatography

[0198] In another embodiment of the present invention, size-exclusion chromatography is used to purify compounds that are bound to a target nucleic acid from a complex mixture of compounds. Size-exclusion chromatography separates molecules based on their size and uses gel-based media comprised of beads with specific size distributions. When applied to a column, this media settles into a tightly packed matrix and forms a complex array of pores. Separation is accomplished by the inclusion or exclusion of molecules by these pores based on molecular size. Small molecules are included into the pores and, consequently, their migration through the matrix is retarded due to the added distance they must travel before elution. Large molecules are excluded from the pores and migrate with the void volume when applied to the matrix. In the present invention, a target nucleic acid is incubated with a mixture of compounds while free in solution and allowed to reach equilibrium. When applied to a size exclusion column, compounds free in solution are retained by the column, and compounds bound to the target nucleic acid are passed through the column. In a preferred embodiment, spin columns commonly used for gel filtration of nucleic acids will be employed to separate bound from unbound compounds (*e.g.*, Bio-Spin columns manufactured by BIO-RAD). In another embodiment, the size exclusion matrix is packed into multiwell plates to allow high throughput separation of mixtures (*e.g.*, PLASMID 96-well SEC plates manufactured by Millipore).

5.4.3.3. Affinity Chromatography

[0199] In one embodiment of the present invention, affinity capture is used to purify compounds that are bound to a target nucleic acid labeled with an affinity tag from a complex mixture of compounds. To accomplish this, a target nucleic acid labeled with an affinity tag is incubated with a mixture of compounds while free in solution and then captured to a solid support once equilibrium has been established; alternatively, target nucleic acids labeled with an affinity tag can be captured to a solid support first and then allowed to reach equilibrium with a mixture of compounds.

[0200] The solid support is typically comprised of, but not limited to, cross-linked agarose beads that are coupled with a ligand for the affinity tag. Alternatively, the solid support may be a glass, silicon, metal, or carbon, plastic (polystyrene, polypropylene) surface with or without a self-assembled monolayer (SAM) either with a covalently

attached ligand for the affinity tag, or with inherent affinity for the tag on the target nucleic acid.

[0201] Once the complex between the target nucleic acid and compound has reached equilibrium and has been captured, one skilled in the art will appreciate that the retention of bound compounds and removal of unbound compounds is facilitated by washing the solid support with large excesses of binding reaction buffer. Furthermore, retention of high affinity compounds and removal of low affinity compounds can be accomplished by a number of means that increase the stringency of washing; these means include, but are not limited to, increasing the number and duration of washes, raising the salt concentration of the wash buffer, addition of detergent or surfactant to the wash buffer, and addition of non-specific competitor to the wash buffer.

[0202] In one embodiment, the compounds themselves are detectably labeled with fluorescent dyes, radioactive isotopes, or nanoparticles. When the compounds are applied to the captured target nucleic acid in a spatially addressed fashion (*e.g.*, in separate wells of a 96-well microplate), binding between the compounds and the target nucleic acid can be determined by the presence of the detectable label on the compound using fluorescence.

[0203] Following the removal of unbound compounds, bound compounds with high affinity for the target nucleic acid can be eluted from the immobilized target nucleic acids and analyzed. The elution of compounds can be accomplished by any means that break the non-covalent interactions between the target nucleic acid and compound. Means for elution include, but are not limited to, changing the pH, changing the salt concentration, the application of organic solvents, and the application of molecules that compete with the bound ligand. In a preferred embodiment, the means employed for elution will release the compound from the target RNA, but will not effect the interaction between the affinity tag and the solid support, thereby achieving selective elution of compound. Moreover, a preferred embodiment will employ an elution buffer that is volatile to allow for subsequent concentration by lyophilization of the eluted compound (e.g., 0 M to 5 M ammonium acetate).

5.5. Methods for Confirming that a Compound Modulates Untranslated Region-Dependent Expression

[0204] In order to exclude the possibility that a particular compound is functioning solely by modulating the expression of a target gene in an untranslated region-independent manner, one or more mutations may be introduced into the untranslated regions operably linked to a reporter gene and the effect on the expression of the reporter gene in a reporter gene-based assay described herein can be determined. For example, a reporter gene

construct comprising the 5' UTR of a target gene may be mutated by deleting a fragment of the 5' UTR of the target gene or substituting a fragment of the 5' UTR of the target gene with a fragment of the 5' UTR of another gene and measuring the expression of the reporter gene in the presence and absence of a compound that has been identified in a screening assays described *supra* (See Section 5.4). If the deletion of a fragment of the 5' UTR of the target gene or the substitution of a fragment of the 5' UTR of the target gene with a fragment of the 5' UTR of another gene affects the ability of the compound to modulate the expression of the reporter gene, then the fragment of the 5' UTR deleted or substituted plays a role in the regulation of the reporter gene expression and the regulation, at least in part, in an untranslated region-dependent manner.

[0205] The possibility that a particular compound is functioning solely by modulating the expression of a target gene in an untranslated region-independent manner may be also determined by changing the vector utilized as a reporter construct. The untranslated regions flanked by a reporter gene from the first reporter construct in which an effect on reporter gene expression was detected following exposure to a compound may be inserted into a new reporter construct that has, *e.g.*, different transcriptional regulation elements (*e.g.*, a different promoter) and a different selectable marker. The level of reporter gene expression in the presence of the compound can be compared to the level of reporter gene expression in the absence of the compound or in the presence of a control (*e.g.*, PBS). If there is no change in the level of expression of the reporter gene in the presence of the compound relative to the absence of the compound or in the presence of a control, then the compound probably is functioning in an untranslated region-independent manner.

[0206] The specificity of a particular compound's effect on untranslated region-dependent expression of a target gene can also be determined. In particular, the effect of a particular compound on the expression of one or more genes (preferably, a plurality of genes) can be determined utilizing assays well-known to one of skill in the art or described herein. In a specific embodiment, the specificity of a particular compound for an untranslated region of a target gene is determined by (a) contacting the compound of interest with a cell containing a nucleic acid comprising a reporter gene operably linked to an UTR of a different gene (*i.e.*, a gene different from the target gene which has a UTR different from the target gene); and (b) detecting a reporter gene protein translated from the reporter gene, wherein the compound is specific for the untranslated region of the target gene if the expression of said reporter gene in the presence of the compound is not altered or is not substantially altered relative to a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS). In

another embodiment, the specificity of a particular compound for an untranslated region of a target gene is determined by (a) contacting the compound of interest with a panel of cells, each cell in a different well of a container (*e.g.*, a 48 or 96 well microtiter plate) and each cell containing a nucleic acid comprising a reporter gene operably linked to an UTR of a different gene; and (b) detecting a reporter gene protein translated from the reporter gene, wherein the compound is specific for the untranslated region of the target gene if the expression of said reporter gene in the presence of the compound is not altered or is not substantially altered relative to a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS). In accordance with this embodiment, the panel may comprise 5, 7, 10, 15, 20, 25, 50, 75, 100 or more cells. In another embodiment, the specificity of a particular compound for an untranslated region of a target gene is determined by (a) contacting the compound of interest with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to an UTR of a different gene; and (b) detecting a reporter gene protein translated from the reporter gene, wherein the compound is specific for the untranslated region of the target gene if the expression of said reporter gene in the presence of the compound is not altered or is not substantially altered relative to a previously determined reference range, or the expression in the absence of the compound or the presence of a control (*e.g.*, PBS). As used herein, the term "not substantially altered" means that the compound alters the expression of the reporter gene or target gene by less than 20%, less than 15%, less than 10%, less than 5%, or less than 2% relative to a negative control such as PBS.

[0207] The compounds identified in the assays described *supra* that modulate untranslated region-dependent expression of a target gene (for convenience referred to herein as a "lead" compound) can be further tested for untranslated region-dependent binding to the target RNA (which contains at least one untranslated region, and preferably at least one element of an untranslated region). Furthermore, by assessing the effect of a compound on target gene expression, *cis*-acting elements, *i.e.*, specific nucleotide sequences, that are involved in untranslated region-dependent expression may be identified.

5.5.1. RNA Binding Assays

[0208] The compounds that modulate untranslated region-dependent expression of a target gene can be tested for binding to the target RNA (which contains at least one untranslated region, and preferably at least one element of an untranslated region) by any method known in the art. See Section 5.4.3 *supra*.

5.5.1. Subtraction Assay

[0209] The element(s) of an untranslated region(s) that is necessary for a compound identified in accordance with the methods of the invention to modulate untranslated region-dependent expression of a target gene can be determined utilizing standard mutagenesis techniques well-known to one of skill in the art. One or more mutations (*e.g.*, deletions, additions and/or substitutions) may be introduced into the untranslated regions operably linked to a reporter gene and the effect on the expression of the reporter gene in a reporter gene-based assay described herein can be determined. For example, a reporter gene construct comprising the 5' UTR of a target gene may be mutated by deleting a fragment or all of the 5' UTR of the target gene or substituting a fragment of the 5' UTR of the target gene with a fragment of the 5' UTR of another gene and measuring the expression of the reporter gene in the presence and absence of a compound that has been identified in a screening assays described *supra* (See Section 5.4). If the deletion of a fragment of the 5' UTR of the target gene or the substitution of a fragment of the 5' UTR of the target gene with a fragment of the 5' UTR of another gene affects the ability of the compound to modulate the expression of the reporter gene, then the fragment of the 5' UTR deleted or substituted plays a role in the regulation of the reporter gene expression.

[0210] Standard techniques known to those of skill in the art can be used to introduce mutations in the nucleotide sequence of an untranslated region of a target gene, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis. In a specific embodiment, less than 75 nucleic acid residue substitutions, less than 50 nucleic acid residue substitutions, less than 45 nucleic acid residue substitutions, less than 40 nucleic acid residue substitutions, less than 35 nucleic acid residue substitutions, less than 30 nucleic acid residue substitutions, less than 25 nucleic acid residue substitutions, less than 20 nucleic acid residue substitutions, less than 15 nucleic acid residue substitutions, less than 10 nucleic acid residue substitutions, or less than 5 nucleic acid residue substitutions are introduced into the nucleotide sequence of an untranslated region of a target gene. In another embodiment, less than 10 elements of an untranslated region of a target gene, less than 9 of an untranslated region of a target gene, less than 8 elements of an untranslated region of a target gene, less than 7 elements of an untranslated region of a target gene, less than 6 elements of an untranslated region of a target gene, less than 5 elements of an untranslated region of a target gene, less than 4 elements of an untranslated region of a target gene, less than 3 elements of an untranslated region of a target gene, or less than 2 elements of an untranslated region of a target gene are mutated at one time.

5.5.3. Expressed Protein Concentration and Activity Assays

[0211] The compounds identified in the reporter gene-based assays described herein that modulate untranslated region-dependent expression may be tested in *in vitro* assays (*e.g.*, cell-free assays) or *in vivo* assays (*e.g.*, cell-based assays) well-known to one of skill in the art or described herein for the effect of said compounds on the expression of the target gene from which the untranslated regions of the reporter gene construct were derived. The specificity of a particular compound's effect on untranslated region-dependent expression of one or more other genes (preferably, a plurality of genes) can also be determined utilizing assays well-known to one of skill in the art or described herein. In a preferred embodiment, a compound identified utilizing the reporter gene-based assays described herein has a specific effect on the expression of only one gene or a group of genes within the same signaling pathway.

[0212] The expression of a gene can be readily detected, *e.g.*, by quantifying the protein and/or RNA encoded by said gene. Many methods standard in the art can be thus employed, including, but not limited to, immunoassays to detect and/or visualize gene expression (*e.g.*, western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunocytochemistry, *etc.*) and/or hybridization assays to detect gene expression by detecting and/or visualizing respectively mRNA encoding a gene (*e.g.*, northern assays, dot blots, *in situ* hybridization, *etc.*). Such assays are routine and well known in the art (see, *e.g.*, Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

[0213] Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasylol) supplemented with protein phosphatase and/or protease inhibitors (*e.g.*, EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell lysate, incubating for a period of time (*e.g.*, 1 to 4 hours) at 40° C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 40° C, washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer. The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, *e.g.*, western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background (*e.g.*, pre-clearing the cell lysate with sepharose beads). For further discussion regarding

immunoprecipitation protocols see, *e.g.*, Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.16.1.

[0214] Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (*e.g.*, 8%- 20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (*e.g.*, PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (*e.g.*, PBS-Tween 20), incubating the membrane with primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, incubating the membrane with a secondary antibody (which recognizes the primary antibody, *e.g.*, an anti-human antibody) conjugated to an enzymatic substrate (*e.g.*, horseradish peroxidase or alkaline phosphatase) or radioactive molecule (*e.g.*, ^{32}P or ^{125}I) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding western blot protocols see, *e.g.*, Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1.

[0215] ELISAs comprise preparing antigen, coating the well of a 96 well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (*e.g.*, horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of the antigen of interest to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs see, *e.g.*, Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1.

[0216] Another antibody based separation that can be used to detect the protein of interest is the use of flow cytometry such as by a florescence activated cell sorter ("FACS"). Typically, separation by flow cytometry is performed as follows. The suspended mixture of

cells are centrifuged and resuspended in media. Antibodies which are conjugated to fluorochrome are added to allow the binding of the antibodies to specific proteins. In another embodiment, the secondary antibodies that are conjugated to fluorochromes can be used to detect primary antibodies specific to the protein of interest. The cell mixture is then washed by one or more centrifugation and resuspension steps. The mixture is run through a FACS which separates the cells based on different fluorescence characteristics. FACS systems are available in varying levels of performance and ability, including multi-color analysis. The facilitating cell can be identified by a characteristic profile of forward and side scatter which is influenced by size and granularity, as well as by positive and/or negative expression of certain cell surface markers.

[0217] In addition to measuring the effect of a compound identified in the reporter gene-based assays described herein on the expression of the target gene from which the untranslated regions of the reporter gene construct were derived, the activity of the protein encoded by the target gene can be assessed utilizing techniques well-known to one of skill in the art. For example, the activity of a protein encoded by a target gene can be determined by detecting induction of a cellular second messenger (*e.g.*, intracellular Ca^{2+}, diacylglycerol, IP_3, *etc.*), detecting the phosphorylation of a protein, detecting the activation of a transcription factor, or detecting a cellular response, for example, cellular differentiation, or cell proliferation. The induction of a cellular second messenger or phosporylation of a protein can be determined by, *e.g.*, immunoassays well-known to one of skill in the art and described herein. The activation of a transcription factor can be detected by, *e.g.*, electromobility shift assays, and a cellular response such as cellular proliferation can be detected by, *e.g.*, trypan blue cell counts, ^{3}H-thymidine incorporation, and flow cytometry.

5.6. Methods for Characterizing the Compounds That Modulate Untranslated Region-Dependent Expression of a Target Gene

[0218] If the library comprises arrays or microarrays of compounds, wherein each compound has an address or identifier, the compound can be deconvoluted, *e.g.*, by cross-referencing the positive sample to original compound list that was applied to the individual test assays.

[0219] If the library is a peptide or nucleic acid library, the sequence of the compound can be determined by direct sequencing of the peptide or nucleic acid. Such methods are well known to one of skill in the art.

[0220] A number of physico-chemical techniques can be used for the *de novo* characterization of compounds bound to the target RNA. Examples of such techniques

include, but are not limited to, mass spectrometry, NMR spectroscopy, X-ray crystallography and vibrational spectroscopy.

5.6.1. Mass Spectrometry

[0221] Mass spectrometry (*e.g.*, electrospray ionization ("ESI"), matrix-assisted laser desorption-ionization ("MALDI"), and Fourier-transform ion cyclotron resonance ("FT-ICR") can be used for elucidating the structure of a compound.

[0222] MALDI uses a pulsed laser for desorption of the ions and a time-of-flight analyzer, and has been used for the detection of noncovalent tRNA:amino-acyl-tRNA synthetase complexes (Gruic-Sovulj et al., 1997, J. Biol. Chem. 272:32084-32091). However, covalent cross-linking between the target nucleic acid and the compound is required for detection, since a non-covalently bound complex may dissociate during the MALDI process.

[0223] ESI mass spectrometry ("ESI-MS") has been of greater utility for studying non-covalent molecular interactions because, unlike the MALDI process, ESI-MS generates molecular ions with little to no fragmentation (Xavier et al., 2000, Trends Biotechnol. 18(8):349-356). ESI-MS has been used to study the complexes formed by HIV Tat peptide and protein with the TAR RNA (Sannes-Lowery et al., 1997, Anal. Chem. 69:5130-5135).

[0224] Fourier-transform ion cyclotron resonance ("FT-ICR") mass spectrometry provides high-resolution spectra, isotope-resolved precursor ion selection, and accurate mass assignments (Xavier et al., 2000, Trends Biotechnol. 18(8):349-356). FT-ICR has been used to study the interaction of aminoglycoside antibiotics with cognate and non-cognate RNAs (Hofstadler et al., 1999, Anal. Chem. 71:3436-3440; and Griffey et al., 1999, Proc. Natl. Acad. Sci. USA 96:10129-10133). As true for all of the mass spectrometry methods discussed herein, FT-ICR does not require labeling of the target RNA or a compound.

[0225] An advantage of mass spectroscopy is not only the elucidation of the structure of the compound, but also the determination of the structure of the compound bound to a target RNA. Such information can enable the discovery of a consensus structure of a compound that specifically binds to a target RNA.

5.6.2. NMR Spectroscopy

[0226] NMR spectroscopy is a valuable technique for identifying complexed target nucleic acids by qualitatively determining changes in chemical shift, specifically from distances measured using relaxation effects, and NMR-based approaches have been used in the identification of small molecule binders of protein drug targets (Xavier et al., 2000,

Trends Biotechnol. 18(8):349-356). The determination of structure-activity relationships ("SAR") by NMR is the first method for NMR described in which small molecules that bind adjacent subsites are identified by two-dimentional ^{1}H-^{15}N spectra of the target protein (Shuker et al., 1996, Science 274:1531-1534). The signal from the bound molecule is monitored by employing line broadening, transferred NOEs and pulsed field gradient diffusion measurements (Moore, 1999, Curr. Opin. Biotechnol. 10:54-58). A strategy for lead generation by NMR using a library of small molecules has been recently described (Fejzo et al., 1999, Chem. Biol. 6:755-769).

[0227] In one embodiment of the present invention, the target nucleic acid complexed to a compound can be determined by SAR by NMR. Furthermore, SAR by NMR can also be used to elucidate the structure of a compound.

[0228] As described above, NMR spectroscopy is a technique for identifying binding sites in target nucleic acids by qualitatively determining changes in chemical shift, specifically from distances measured using relaxation effects. Examples of NMR that can be used for the invention include, but are not limited to, one-dimentional NMR, two-dimentional NMR, correlation spectroscopy ("COSY"), and nuclear Overhauser effect ("NOE") spectroscopy. Such methods of structure determination of compounds are well-known to one of skill in the art.

[0229] Similar to mass spectroscopy, an advantage of NMR is the not only the elucidation of the structure of the compound, but also the determination of the structure of the compound bound to the target RNA. Such information can enable the discovery of a consensus structure of a compound that specifically binds to a target RNA.

5.6.3. X ray Crystallography

[0230] X-ray crystallography can be used to elucidate the structure of a compound. For a review of x-ray crystallography see, *e.g.*, Blundell et al., 2002, Nat Rev Drug Discov 1(1):45-54. The first step in x-ray crystallography is the formation of crystals. The formation of crystals begins with the preparation of highly purified and soluble samples. The conditions for crystallization are then determined by optimizing several solution variables known to induce nucleation, such as pH, ionic strength, temperature, and specific concentrations of organic additives, salts and detergent. Techniques for automating the crystallization process have been developed for the production of high-quality protein crystals. Once crystals have been formed, the crystals are harvested and prepared for data collection. The crystals are then analyzed by diffraction (such as multi-circle diffractometers, high-speed CCD detectors, and detector off-set). Generally, multiple crystals must be screened for structure determinations.

5.6.4. Vibrational Spectroscopy

[0231] Vibrational spectroscopy (*e.g.* infrared (IR) spectroscopy or Raman spectroscopy) can be used for elucidating the structure of a compound.

[0232] Infrared spectroscopy measures the frequencies of infrared light (wavelengths from 100 to 10,000 nm) absorbed by the compound as a result of excitation of vibrational modes according to quantum mechanical selection rules which require that absorption of light cause a change in the electric dipole moment of the molecule. The infrared spectrum of any molecule is a unique pattern of absorption wavelengths of varying intensity that can be considered as a molecular fingerprint to identify any compound.

[0233] Infrared spectra can be measured in a scanning mode by measuring the absorption of individual frequencies of light, produced by a grating which separates frequencies from a mixed-frequency infrared light source, by the compound relative to a standard intensity (double-beam instrument) or pre-measured ('blank') intensity (single-beam instrument). In a preferred embodiment, infrared spectra are measured in a pulsed mode ("FT-IR") where a mixed beam, produced by an interferometer, of all infrared light frequencies is passed through or reflected off the compound. The resulting interferogram, which may or may not be added with the resulting interferograms from subsequent pulses to increase the signal strength while averaging random noise in the electronic signal, is mathematically transformed into a spectrum using Fourier Transform or Fast Fourier Transform algorithms.

[0234] Raman spectroscopy measures the difference in frequency due to absorption of infrared frequencies of scattered visible or ultraviolet light relative to the incident beam. The incident monochromatic light beam, usually a single laser frequency, is not truly absorbed by the compound but interacts with the electric field transiently. Most of the light scattered off the sample will be unchanged (Rayleigh scattering) but a portion of the scatter light will have frequencies that are the sum or difference of the incident and molecular vibrational frequencies. The selection rules for Raman (inelastic) scattering require a change in polarizability of the molecule. While some vibrational transitions are observable in both infrared and Raman spectrometry, must are observable only with one or the other technique. The Raman spectrum of any molecule is a unique pattern of absorption wavelengths of varying intensity that can be considered as a molecular fingerprint to identify any compound.

[0235] Raman spectra are measured by submitting monochromatic light to the sample, either passed through or preferably reflected off, filtering the Rayleigh scattered light, and detecting the frequency of the Raman scattered light. An improved Raman spectrometer is

described in US Patent No. 5,786,893 to Fink et al., which is hereby incorporated by reference.

[0236] Vibrational microscopy can be measured in a spatially resolved fashion to address single beads by integration of a visible microscope and spectrometer. A microscopic infrared spectrometer is described in U.S. Patent No. 5,581,085 to Reffner et al., which is hereby incorporated by reference in its entirety. An instrument that simultaneously performs a microscopic infrared and microscopic Raman analysis on a sample is described in U.S. Patent No. 5,841,139 to Sostek et al., which is hereby incorporated by reference in its entirety.

[0237] In one embodiment of the method, compounds are synthesized on polystyrene beads doped with chemically modified styrene monomers such that each resulting bead has a characteristic pattern of absorption lines in the vibrational (IR or Raman) spectrum, by methods including but not limited to those described by Fenniri et al., 2000, J. Am. Chem. Soc. 123:8151-8152. Using methods of split-pool synthesis familiar to one of skill in the art, the library of compounds is prepared so that the spectroscopic pattern of the bead identifies one of the components of the compound on the bead. Beads that have been separated according to their ability to bind target RNA can be identified by their vibrational spectrum. In one embodiment of the method, appropriate sorting and binning of the beads during synthesis then allows identification of one or more further components of the compound on any one bead. In another embodiment of the method, partial identification of the compound on a bead is possible through use of the spectroscopic pattern of the bead with or without the aid of further sorting during synthesis, followed by partial resynthesis of the possible compounds aided by doped beads and appropriate sorting during synthesis.

[0238] In another embodiment, the IR or Raman spectra of compounds are examined while the compound is still on a bead, preferably, or after cleavage from bead, using methods including but not limited to photochemical, acid, or heat treatment. The compound can be identified by comparison of the IR or Raman spectral pattern to spectra previously acquired for each compound in the combinatorial library.

5.7. Secondary Screens of Compounds

[0239] Once a compound has been identified to modulate untranslated region-dependent expression of a target gene and preferably, the structure of the compound has been identified by the methods described in Section 5.6, the compounds are tested for biological activity in further assays and/or animal models (see, *e.g.,* Sections 5.7.1 and 5.7.2). Further, a lead compound may be used to design congeners or analogs (see, *e.g.*, Section 5.7.3).

5.7.1. Cell-based Screens

[0240] The compounds identified in the assays described *supra* (for convenience referred to herein as a "lead" compound) can be tested for biological activity using host cells containing or engineered to contain the target RNA element involved in untranslated region-dependent gene expression coupled to a functional readout system. For example, a phenotypic or physiological readout can be used to assess untranslated region-dependent activity of the target RNA in the presence and absence of the lead compound.

[0241] In one embodiment, a phenotypic or physiological readout can be used to assess untranslated region-dependent activity of the target RNA in the presence and absence of the lead compound. For example, the target RNA may be overexpressed in a cell in which the target RNA is endogenously expressed. Where the target RNA controls untranslated region-dependent expression of a gene product involved in cell growth or viability, the *in vivo* effect of the lead compound can be assayed by measuring the cell growth or viability of the target cell. Such assays can be carried out with representative cells of cell types involved in a particular disease or disorder (*e.g.*, leukocytes such as T cells, B cells, natural killer cells, macrophages, neutrophils and eosinophils). A lower level of proliferation or survival of the contacted cells indicates that the lead compound is effective to treat a condition in the patient characterized by uncontrolled cell growth. Alternatively, instead of culturing cells from a patient, a lead compound may be screened using cells of a tumor or malignant cell line or an endothelial cell line. Specific examples of cell culture models include, but are not limited to, for lung cancer, primary rat lung tumor cells (see, *e.g.*, Swafford et al., 1997, Mol. Cell. Biol., 17:1366-1374) and large-cell undifferentiated cancer cell lines (see, *e.g.*, Mabry et al., 1991, Cancer Cells, 3:53-58); colorectal cell lines for colon cancer (see, *e.g.*, Park & Gazdar, 1996, J. Cell Biochem. Suppl. 24:131-141); multiple established cell lines for breast cancer (see, *e.g.*, Hambly et al., 1997, Breast Cancer Res. Treat. 43:247-258; Gierthy et al., 1997, Chemosphere 34:1495-1505; and Prasad & Church, 1997, Biochem. Biophys. Res. Commun. 232:14-19); a number of well-characterized cell models for prostate cancer (see, *e.g.*, Webber et al., 1996, Prostate, Part 1, 29:386-394; Part 2, 30:58-64; and Part 3, 30:136-142 and Boulikas, 1997, Anticancer Res. 17:1471-1505); for genitourinary cancers, continuous human bladder cancer cell lines (see, *e.g.*, Ribeiro et al., 1997, Int. J. Radiat. Biol. 72:11-20); organ cultures of transitional cell carcinomas (see, *e.g.*, Booth et al., 1997, Lab Invest. 76:843-857) and rat progression models (see, *e.g.*, Vet et al., 1997, Biochim. Biophys Acta 1360:39-44); and established cell lines for leukemias and lymphomas (see, *e.g.*, Drexler, 1994, Leuk. Res. 18:919-927 and Tohyama, 1997, Int. J. Hematol. 65:309-317).

[0242] Many assays well-known in the art can be used to assess the survival and/or growth of a patient cell or cell line following exposure to a lead compound; for example, cell proliferation can be assayed by measuring bromodeoxyuridine (BrdU) incorporation (see, *e.g.*, Hoshino et al., 1986, Int. J. Cancer 38:369 and Campana et al., 1988, J. Immunol. Meth. 107:79) or (^{3}H)-thymidine incorporation (see, *e.g.*, Chen, 1996, Oncogene 13:1395-403 and Jeoung, 1995, J. Biol. Chem. 270:18367-73), by direct cell count, by detecting changes in transcription, translation or activity of known genes such as proto-oncogenes (*e.g.*, *fos, myc*) or cell cycle markers (Rb, cdc2, cyclin A, D1, D2, D3, E, *etc.*). The levels of such protein and mRNA and activity can be determined by any method well known in the art. For example, protein can be quantitated by known immunodiagnostic methods such as western blotting or immunoprecipitation using commercially available antibodies. mRNA can be quantitated using methods that are well known and routine in the art, for example, using northern analysis, RNase protection, the polymerase chain reaction in connection with reverse transcription ("RT-PCR"). Cell viability can be assessed by using trypan-blue staining or other cell death or viability markers known in the art. In a specific embodiment, the level of cellular ATP is measured to determined cell viability. Differentiation can be assessed, for example, visually based on changes in morphology.

[0243] The lead compound can also be assessed for its ability to inhibit cell transformation (or progression to malignant phenotype) *in vitro*. In this embodiment, cells with a transformed cell phenotype are contacted with a lead compound, and examined for change in characteristics associated with a transformed phenotype (a set of *in vitro* characteristics associated with a tumorigenic ability *in vivo*), for example, but not limited to, colony formation in soft agar, a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, or expression of fetal antigens, *etc.* (see, *e.g.*, Luria et al., 1978, General Virology, 3d Ed., John Wiley & Sons, New York, pp. 436-446).

[0244] Loss of invasiveness or decreased adhesion can also be assessed to demonstrate the anti-cancer effects of a lead compound. For example, an aspect of the formation of a metastatic cancer is the ability of a precancerous or cancerous cell to detach from primary site of disease and establish a novel colony of growth at a secondary site. The ability of a cell to invade peripheral sites reflects its potential for a cancerous state. Loss of invasiveness can be measured by a variety of techniques known in the art including, for example, induction of E-cadherin-mediated cell-cell adhesion. Such E-cadherin-mediated

adhesion can result in phenotypic reversion and loss of invasiveness (see, *e.g.*, Hordijk et al., 1997, Science 278:1464-66).

[0245] Loss of invasiveness can further be examined by inhibition of cell migration. A variety of 2-dimensional and 3-dimensional cellular matrices are commercially available (Calbiochem-Novabiochem Corp. San Diego, CA). Cell migration across or into a matrix can be examined using microscopy, time-lapsed photography or videography, or by any method in the art allowing measurement of cellular migration. In a related embodiment, loss of invasiveness is examined by response to hepatocyte growth factor ("HGF"). HGF-induced cell scattering is correlated with invasiveness of cells such as Madin-Darby canine kidney ("MDCK") cells. This assay identifies a cell population that has lost cell scattering activity in response to HGF (see, *e.g.*, Hordijk et al., 1997, Science 278:1464-66).

[0246] Alternatively, loss of invasiveness can be measured by cell migration through a chemotaxis chamber (Neuroprobe/ Precision Biochemicals Inc. Vancouver, BC). In such assay, a chemo-attractant agent is incubated on one side of the chamber (*e.g.*, the bottom chamber) and cells are plated on a filter separating the opposite side (*e.g.*, the top chamber). In order for cells to pass from the top chamber to the bottom chamber, the cells must actively migrate through small pores in the filter. Checkerboard analysis of the number of cells that have migrated can then be correlated with invasiveness (see *e.g.*, Ohnishi, 1993, Biochem. Biophys. Res. Commun.193:518-25).

[0247] The effect of a compound of the invention on cell adhesion can be measured using HUVECS. HUVECs are seeded on 24 well culture plates and incubated for 2 days to allow formation of a confluent monolayer. Cancerous cells or a cancer cell line such as LS-180 human colon adenocarcinoma cells are labeled with 5 μM Calcein-AM for 30 min. Calcein-AM labeled LS180 cells are added into each well of the HUVEC culture; and incubated for 10 min at 37^0C. TNF-α (80 ng/ml) is then added and the culture incubated for is an additional 110 min. Non-adherent cells are removed by washing with PBS. The fluorescence intensity of adherent LS-180 cell in each individual well is measured by a fluorescent plate reader set at excitation 485/20 nm and emission at 530/25 nm.

[0248] The effect of a compound of the invention on cell migration and invasion can also be determined using an assay based on the BD BioCoast Angiogenesis System (BD Biosciences, Bedford, MA). The fluorescence blocking membrane of the insert is a 3 micron pore size PET filter which has been coated either with BD Matrigel basement matrix (for invasion assay) or without Matrigel matrix (for migration assay). HUVECs (250 μl/well) in culture medium without serum are added to the top chamber; a compound added to bottom wells containing medium (750 μl/well) with VEGF as a chemo-attractant. Cells

are then incubated for 22 h at 37°C. After incubation, cells are stained with Calcein AM for measurement of fluorescence.

[0249] Further, a lead compound can be assessed for its ability to alter viral replication (as determined, *e.g.*, by plaque formation) or the production of viral proteins (as determined, *e.g.*, by western blot analysis) or viral RNAs (as determined, *e.g.*, by RT-PCR or northern blot analysis) in cultured cells *in vitro* using methods which are well known in the art. A lead compound can also be assessed for its ability to alter bacterial replication (as determined, *e.g.*, by measuring bacterial growth rates) or the production of bacterial proteins (as determined, *e.g.*, by western blot analysis) or bacterial RNAs (as determined, *e.g.*, by RT-PCR or northern blot analysis) in cultured cells *in vitro* using methods which are well known in the art. Finally, a lead compound can be assessed for its ability to alter fungal replication (as determined, *e.g.*, by fungal growth rates, such as macrodilution methods and/or microdilution methods using protocols known to those skilled in the art (see, *e.g.*, Clancy et al., 1997, Journal of Clinical Microbiology, 35(11): 2878-82; Ryder et al., 1998, Antimicrobial Agents and Chemotherapy, 42(5): 1057-61; or U.S. Patent Nos. 5,521,153; U.S. 5,883,120, U.S. 5,521,169)) or the production of fungal proteins (as determined, *e.g.*, by western blot analysis) or fungal RNAs (as determined, *e.g.*, by RT-PCR or northern blot analysis) in cultured cells *in vitro* using methods which are well known in the art.

5.7.2. Animal Model-based Screens

[0250] The lead compounds identified in the reporter gene-based assay described herein can be tested for biological activity using animal models for a disease, disorder, condition, or syndrome of interest. These include animals engineered to contain a target gene coupled to a functional readout system, such as a transgenic mouse. Such animal model systems include, but are not limited to, rats, mice, chicken, cows, monkeys, pigs, dogs, rabbits, *etc.* In a specific embodiment of the invention, a compound identified in accordance with the methods of the invention is tested in a mouse model system. Such model systems are widely used and well-known to the skilled artisan such as the SCID mouse model or transgenic mice.

[0251] The anti-angiogenic activity of a compound identified in accordance with the invention can be determined by using various experimental animal models of vascularized tumors. The anti-tumor activity of a compound identified in accordance with the invention can be determined by administering the compound to an animal model and verifying that the compound is effective in reducing the proliferation or spread of cancer cells in said animal model. An example of an animal model for human cancer in general includes, but is

not limited to, spontaneously occurring tumors of companion animals (see, *e.g.*, Vail & MacEwen, 2000, Cancer Invest 18(8):781-92).

[0252] Examples of animal models for lung cancer include, but are not limited to, lung cancer animal models described by Zhang & Roth (1994, In Vivo 8(5):755-69) and a transgenic mouse model with disrupted p53 function (see, *e.g.*, Morris et al., 1998, J La State Med Soc 150(4):179-85). An example of an animal model for breast cancer includes, but is not limited to, a transgenic mouse that overexpresses cyclin D1 (see, *e.g.*, Hosokawa et al., 2001, Transgenic Res 10(5):471-8). An example of an animal model for colon cancer includes, but is not limited to, a TCR b and p53 double knockout mouse (see, *e.g.*, Kado et al., 2001, Cancer Res 61(6):2395-8). Examples of animal models for pancreatic cancer include, but are not limited to, a metastatic model of Panc02 murine pancreatic adenocarcinoma (see, *e.g.*, Wang et al., 2001, Int J Pancreatol 29(1):37-46) and nu-nu mice generated in subcutaneous pancreatic tumours (see, *e.g.*, Ghaneh et al., 2001, Gene Ther 8(3):199-208). Examples of animal models for non-Hodgkin's lymphoma include, but are not limited to, a severe combined immunodeficiency ("SCID") mouse (see, *e.g.*, Bryant et al., 2000, Lab Invest 80(4):553-73) and an IgHmu-HOX11 transgenic mouse (see, *e.g.*, Hough et al., 1998, Proc Natl Acad Sci USA 95(23):13853-8). An example of an animal model for esophageal cancer includes, but is not limited to, a mouse transgenic for the human papillomavirus type 16 E7 oncogene (see, *e.g.*, Herber et al., 1996, J Virol 70(3):1873-81). Examples of animal models for colorectal carcinomas include, but are not limited to, Apc mouse models (see, *e.g.*, Fodde & Smits, 2001, Trends Mol Med 7(8):369-73 and Kuraguchi et al., 2000, Oncogene 19(50):5755-63).

[0253] The anti-inflammatory activity of a compound identified in accordance with the invention can be determined by using various experimental animal models of inflammatory arthritis known in the art and described in Crofford & Wilder, "Arthritis and Autoimmunity in Animals", in Arthritis and Allied Conditions: A Textbook of Rheumatology, McCarty et al.(eds.), Chapter 30 (Lee & Febiger, 1993). Experimental and spontaneous animal models of inflammatory arthritis and autoimmune rheumatic diseases can also be used to assess the anti-inflammatory activity of a compound identified in accordance with the invention. The principle animal models for arthritis or inflammatory disease known in the art and widely used include: adjuvant-induced arthritis rat models, collagen-induced arthritis rat and mouse models and antigen-induced arthritis rat, rabbit and hamster models, all described in Crofford & Wilder, "Arthritis and Autoimmunity in Animals", in Arthritis and Allied Conditions: A Textbook of Rheumatology, McCarty et al.(eds.), Chapter 30 (Lee & Febiger, 1993), incorporated herein by reference in its entirety.

[0254] The anti-inflammatory activity of a compound identified in accordance with the invention can be assessed using a carrageenan-induced arthritis rat model. Carrageenan-induced arthritis has also been used in rabbit, dog and pig in studies of chronic arthritis or inflammation. Quantitative histomorphometric assessment is used to determine therapeutic efficacy. The methods for using such a carrageenan-induced arthritis model is described in Hansra et al., 2000, Inflammation, 24(2): 141-155. Also commonly used are zymosan-induced inflammation animal models as known and described in the art.

[0255] The anti-inflammatory activity of a compound identified in accordance with the invention can also be assessed by measuring the inhibition of carrageenan-induced paw edema in the rat, using a modification of the method described in Winter et al., 1962, Proc. Soc. Exp. Biol Med. 111, 544-547. This assay has been used as a primary *in vivo* screen for the anti-inflammatory activity of most NSAIDs, and is considered predictive of human efficacy. The anti-inflammatory activity of a compound identified in accordance with the invention is expressed as the percent inhibition of the increase in hind paw weight of the test group relative to the vehicle dosed control group.

[0256] In a specific embodiment of the invention where the experimental animal model used is adjuvant-induced arthritis rat model, body weight can be measured relative to a control group to determine the anti-inflammatory activity of a compound identified in accordance with the invention. Alternatively, the efficacy of a compound identified in accordance with the invention can be assessed using assays that determine bone loss. Animal models such as ovariectomy-induced bone resorption mice, rat and rabbit models are known in the art for obtaining dynamic parameters for bone formation. Using methods such as those described by Yositake et al. or Yamamoto et al., bone volume is measured *in vivo* by microcomputed tomography analysis and bone histomorphometry analysis (see, *e.g.*, Yoshitake et al., 1999, Proc. Natl. Acad. Sci. 96:8156-8160 and Yamamoto et al., 1998, Endocrinology 139(3):1411-1419, both incorporated herein by reference in their entireties).

[0257] Additionally, animal models for inflammatory bowel disease can also be used to assess the efficacy of a compound identified in accordance with the invention (see, *e.g.*, Kim et al., 1992, Scand. J. Gastroentrol. 27:529-537 and Strober, 1985, Dig. Dis. Sci. 30(12 Suppl):3S-10S). Ulcerative cholitis and Crohn's disease are human inflammatory bowel diseases that can be induced in animals. Sulfated polysaccharides including, but not limited to, amylopectin, carrageen, amylopectin sulfate, and dextran sulfate or chemical irritants including, but not limited to, trinitrobenzenesulphonic acid (TNBS) and acetic acid can be administered to animals orally to induce inflammatory bowel diseases.

[0258] Animal models for asthma can also be used to assess the efficacy of a compound identified in accordance with the invention. An example of one such model is the murine adoptive transfer model in which aeroallergen provocation of TH1 or TH2 recipient mice results in TH effector cell migration to the airways and is associated with an intense neutrophilic (TH1) and eosinophilic (TH2) lung mucosal inflammatory response (see, *e.g.*, Cohn et al., 1997, J. Exp. Med. 1861737-1747).

[0259] Animal models for autoimmune disorders can also be used to assess the efficacy of a compound identified in accordance with the invention. Animal models for autoimmune disorders such as type 1 diabetes, thyroid autoimmunity, sytemic lupus eruthematosus, and glomerulonephritis have been developed (see, *e.g.*, Flanders et al., 1999, Autoimmunity 29:235-246; Krogh et al., 1999, Biochimie 81:511-515; and Foster, 1999, Semin. Nephrol. 19:12-24).

[0260] Animal models for viral infections can also be used to assess the efficacy of a compound identified in accordance with the invention. Animal models for viral infections such as EBV-associated diseases, gammaherpesviruses, infectious mononucleosis, simian immunodeficiency virus ("SIV"), Borna disease virus infection, hepatitis, varicella virus infection, viral pneumonitis, Epstein-Barr virus pathogenesis, feline immunodeficiency virus ("FIV"), HTLV type 1 infection, human rotaviruses, and genital herpes have been developed (see, *e.g.*, Hayashi et al., 2002, Histol Histopathol 17(4):1293-310; Arico et al., 2002, J Interferon Cytokine Res 22(11):1081-8; Flano et al., 2002, Immunol Res 25(3):201-17; Sauermann, 2001, Curr Mol Med 1(4):515-22; Pletnikov et al., 2002, Front Biosci 7:d593-607; Engler et al., 2001, Mol Immunol 38(6):457-65; White et al., 2001, Brain Pathol 11(4):475-9; Davis & Matalon, 2001, News Physiol Sci 16:185-90; Wang, 2001, Curr Top Microbiol Immunol. 258:201-19; Phillips et al., 2000, J Psychopharmacol. 14(3):244-50; Kazanji, 2000, AIDS Res Hum Retroviruses. 16(16):1741-6; Saif et al., 1996, Arch Virol Suppl. 12:153-61; and Hsiung et al., 1984, Rev Infect Dis. 6(1):33-50).

[0261] Animal models for bacterial infections can also be used to assess the efficacy of a compound identified in accordance with the invention. Animal models for bacterial infections such as *H. pylori*-infection, genital mycoplasmosis, primary sclerosing cholangitis, cholera, chronic lung infection with *Pseudomonas aeruginosa*, Legionnaires' disease, gastroduodenal ulcer disease, bacterial meningitis, gastric *Helicobacter* infection, pneumococcal otitis media, experimental allergic neuritis, leprous neuropathy, mycobacterial infection, endocarditis, Aeromonas-associated enteritis, *Bacteroides fragilis* infection, syphilis, streptococcal endocarditis, acute hematogenous osteomyelitis, human scrub typhus, toxic shock syndrome, anaerobic infections, *Escherichia coli* infections, and

Mycoplasma pneumoniae infections have been developed (see, *e.g.*, Sugiyama et al., 2002, J Gastroenterol. 37 Suppl 13:6-9; Brown et al., 2001, Am J Reprod Immunol. 46(3):232-41; Vierling, 2001, Best Pract Res Clin Gastroenterol. 15(4):591-610; Klose, 2000, Trends Microbiol. 8(4):189-91; Stotland et al., 2000, Pediatr Pulmonol. 30(5):413-24; Brieland et al., 2000, Immunopharmacology 48(3):249-52; Lee, 2000, Baillieres Best Pract Res Clin Gastroenterol. 14(1):75-96; Koedel & Pfister, 1999, Infect Dis Clin North Am. 13(3):549-77; Nedrud, 1999, FEMS Immunol Med Microbiol. 24(2):243-50; Prellner et al., 1999, Microb Drug Resist. 5(1):73-82; Vriesendorp, 1997, J Infect Dis. 176 Suppl 2:S164-8; Shetty & Antia, 1996, Indian J Lepr. 68(1):95-104; Balasubramanian et al., 1994, Immunobiology 191(4-5):395-401; Carbon et al., 1994, Int J Biomed Comput. 36(1-2):59-67; Haberberger et al., 1991, Experientia. 47(5):426-9; Onderdonk et al., 1990, Rev Infect Dis. 12 Suppl 2:S169-77; Wicher & Wicher, 1989, Crit Rev Microbiol. 16(3):181-234; Scheld, 1987, J Antimicrob Chemother. 20 Suppl A:71-85; Emslie & Nade, 1986, Rev Infect Dis. 8(6):841-9; Ridgway et al., 1986, Lab Anim Sci. 36(5):481-5; Quimby & Nguyen, 1985, Crit Rev Microbiol. 12(1):1-44; Onderdonk et al., 1979, Rev Infect Dis. 1(2):291-301; Smith, 1976, Ciba Found Symp. (42):45-72, and Taylor-Robinson, 1976, Infection. 4(1 Suppl):4-8).

[0262] Animal models for fungal infections can also be used to assess the efficacy of a compound identified in accordance with the invention. Animal models for fungal infections such as *Candida* infections, zygomycosis, *Candida* mastitis, progressive disseminated trichosporonosis with latent trichosporonemia, disseminated candidiasis, pulmonary paracoccidioidomycosis, pulmonary aspergillosis, *Pneumocystis carinii* pneumonia, cryptococcal meningitis, coccidioidal meningoencephalitis and cerebrospinal vasculitis, *Aspergillus* niger infection, *Fusarium* keratitis, paranasal sinus mycoses, Aspergillus fumigatus endocarditis, tibial dyschondroplasia, *Candida glabrata* vaginitis, oropharyngeal candidiasis, X-linked chronic granulomatous disease, tinea pedis, cutaneous candidiasis, mycotic placentitis, disseminated trichosporonosis, allergic bronchopulmonary aspergillosis, mycotic keratitis, *Cryptococcus neoformans* infection, fungal peritonitis, *Curvularia* geniculata infection, staphylococcal endophthalmitis, sporotrichosis, and dermatophytosis have been developed (see, *e.g.*, Arendrup et al., 2002, Infection 30(5):286-91; Kamei, 2001, Mycopathologia 152(1):5-13; Guhad et al., 2000, FEMS Microbiol Lett.192(1):27-31; Yamagata et al., 2000, J Clin Microbiol. 38(9):32606; Andrutis et al., 2000, J Clin Microbiol. 38(6):2317-23; Cock et al., 2000, Rev Inst Med Trop Sao Paulo 42(2):59-66; Shibuya et al., 1999, Microb Pathog. 27(3):123-31; Beers et al., 1999, J Lab Clin Med. 133(5):423-33; Najvar et al., 1999, Antimicrob Agents Chemother.43(2):413-4;

Williams et al., 1988, J Infect Dis. 178(4):1217-21; Yoshida, 1988, Kansenshogaku Zasshi. 1998 Jun;72(6):621-30; Alexandrakis et al., 1998, Br J Ophthalmol. 82(3):306-11; Chakrabarti et al., 1997, J Med Vet Mycol. 35(4):295-7; Martin et al., 1997, Antimicrob Agents Chemother. 41(1):13-6; Chu et al., 1996, Avian Dis. 40(3):715-9; Fidel et al., 1996, J Infect Dis. 173(2):425-31; Cole et al., 1995, FEMS Microbiol Lett. 15;126(2):177-80; Pollock et al., 1995, Nat Genet. 9(2):202-9; Uchida et al., 1994, Jpn J Antibiot. 47(10):1407-12; : Maebashi et al., 1994, J Med Vet Mycol. 32(5):349-59; Jensen & Schonheyder, 1993, J Exp Anim Sci. 35(4):155-60; Gokaslan & Anaissie, 1992, Infect Immun. 60(8):3339-44; Kurup et al., 1992, J Immunol. 148(12):3783-8; Singh et al., 1990, Mycopathologia. 112(3):127-37; Salkowski & Balish, 1990, Infect Immun. 58(10):3300-6; Ahmad et al., 1986, Am J Kidney Dis. 7(2):153-6; Alture-Werber E, Edberg SC, 1985, Mycopathologia. 89(2):69-73; Kane et al., 1981, Antimicrob Agents Chemother. 20(5):595-9; Barbee et al., 1977, Am J Pathol. 86(1):281-4; and Maestrone et al., 1973, Am J Vet Res. 34(6):833-6).

[0263] The toxicity and/or efficacy of a compound identified in accordance with the invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. A compound identified in accordance with the invention that exhibits large therapeutic indices is preferred. While a compound identified in accordance with the invention that exhibits toxic side effects may be used, care should be taken to design a delivery system that targets such agents to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0264] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage of a compound identified in accordance with the invention for use in humans. The dosage of such agents lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any agent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately

are involved in a common process or processes in post-transcriptional control of gene expression. If functional involvement in post-transcriptional control of gene expression has been reported for any of the untranslated regions in the set, the untranslated regions without known roles in gene expression regulation can be assigned a function. By performing further analysis and looking for sets of compounds that modulate sets of untranslated regions common to a particular pathological condition, the following can be identified: (a) members of biochemical reaction pathways involved in the disease, (b) targets for multiple drug intervention and/or regulation, and (c) multiple pathological conditions that can be treated with a single compound or set of compounds.

6. EXAMPLE: THERAPEUTIC UNTRANSLATED REGION TARGETS

[0337] The therapeutic targets presented herein are by way of example, and the present invention is not to be limited by the targets described herein. The therapeutic targets presented herein as DNA sequences are understood by one of skill in the art that the sequences can be converted to RNA sequences.

6.1. Tumor Necrosis Factor Alpha

[0338] See, *e.g.*, GenBank Accession # X01394.

General Target Regions:

[0339] (1) 5' Untranslated Region - nts 1 - 152 of GenBank Accession # X01394:

gcagaggacc agctaagagg gagagaagca actacagacc ccccctgaaa acaaccctca gacgccacat ccccctgacaa gctgccaggc aggttctctt cctctcacat actgacccac ggctccaccc tctctccccct ggaaaggaca cc (SEQ ID NO: 5)

[0340] (2) 3' Untranslated Region - nts 852 - 1643 of GenBank Accession # X01394:

tgaggagga cgaacatcca accttcccaa acgcctcccc tgccccaatc cctttattac cccctccttc agacaccctc aacctcttct ggctcaaaaa gagaattggg ggcttagggt cggaacccaa gcttagaact ttaagcaaca agaccaccac ttcgaaacct gggattcagg aatgtgtggc ctgcacagtg aattgctggc aaccactaag aattcaaact ggggcctcca gaactcactg gggcctacag ctttgatccc tgacatctgg aatctggaga ccagggagcc tttggttctg gccagaatgc tgcaggactt gagaagacct cacctagaaa ttgacacaag tggaccttag gccttcctct ctccagatgt ttccagactt ccttgagaca cggagcccag ccctccccat ggagccagct ccctctattt atgtttgcac ttgtgattat ttattattta tttattattt atttatttac agatgaatgt atttatttgg gagaccgggg tatcctgggg gacccaatgt aggagctgcc ttggctcaga catgttttcc gtgaaaacgg agctgaacaa taggctgttc ccatgtagcc ccctggcctc tgtgccttct tttgattatg ttttttaaaa tatttatctg attaagttgt ctaaacaatg ctgatttggt gaccaactgt cactcattgc tgagcctctg ctccccaggg gagttgtgtc tgtaatcgcc ctactattca gtggcgagaa ataaagtttg ctt (SEQ ID NO: 6)

Initial Specific Target Motif:

ccttgtgcac tgcagacctg gtactcagat tttgctatga ggttaatgaa gtaccaagct gtgcttgaat aacgatatgt tttctcagat tttctgttgt acagtttaat ttagcagtcc atatcacatt gcaaaagtag caatgacctc ataaaatacc tcttcaaaat gcttaaattc atttcacaca ttaattttat ctcagtcttg aagccaattc agtaggtgca ttggaatcaa gcctggctac ctgcatgctg ttccttttct tttcttcttt tagccatttt gctaagagac acagtcttct caaacacttc gtttctccta ttttgtttta ctagttttaa gatcagagtt cactttcttt ggactctgcc tatattttct tacctgaact tttgcaagtt ttcaggtaaa cctcagctca ggactgctat ttagctcctc ttaagaagat taaaaaaaaa aaaaaa (SEQ ID NO: 66)

6.28. Her-2

[0430] General Target Regions:

[0431] (1) 5' Untranslated Region:

gcgcccggcccccaccctcgcagcaccccgcgccccgcgccctcccagccgggtccagccggagccatggggccggagcc gcagtgagcaccatggag (SEQ ID NO: 67)

[0432] (2) 3' Untranslated Region:

tgaaccagaaggccaagtccgcagaagccctgatgtgtcctcagggagcagggaaggcctgacttctgctggcatcaagaggtg ggagggccctccgaccacttccaggggaacctgccatgccaggaacctgtcctaaggaaccttccttcctgcttgagttcccagatg gctggaaggggtccagcctcgttggaagaggaacagcactggggagtctttgtggattctgaggccctgcccaatgagactctagg gtccagtggatgccacagcccagcttggccctttccttccagatcctgggtactgaaagccttagggaagctggcctgagagggga agcggccctaagggagtgtctaagaacaaaagcgacccattcagagactgtccctgaaacctagtactgccccccatgaggaagg aacagcaatggtgtcagtatccaggctttgtacagagtgcttttctgtttagtttttacttttttgttttgttttttttaaagacgaaataaagac ccaggggagaatgggtgttgtatggggaggcaagtgtgggggtccttctccacacccactttgtccatttgcaaatatattttggaaa ac (SEQ ID NO: 68)

7. EXAMPLE: VASCULAR ENDOTHELIAL GROWTH FACTOR

7.1. Introduction

[0433] Vascular endothelial growth factor (VEGF) plays a key role in tumor angiogenesis. Considerable evidence demonstrates that VEGF is a viable target for tumor therapy (Carmeliet & Jain, 2000, Nature 407:249-257; Sepp-Lorenzino & Pan, 2000, Angiogenesis – Research frontiers. A basic science conference of the New York Academy of Medicine. Exp. Opin. Invest. Drugs 9:1-7; and Hichlin et al., 2001, DDT 6: 517-528). There are several ongoing clinical trials (phase I-phase III) indicating that either VEGF neutralizing antibodies or VEGFR2-mediated signal transduction inhibitors are effective for tumor therapy (Carmeliet & Jain, 2000, Nature 407:249-257 and Matter, 2001, Drug Discovery Today 6:1005-1024).

[0434] VEGF protein expression is tightly regulated at both the transcriptional and post-transcriptional levels. Under hypoxic conditions, tumor cells express high levels of VEGF that can promote angiogenesis and thus support the growth of tumor cells. Increase of

VEGF protein is due to both increased transcription and enhanced mRNA stability. Hypoxia-inducible factor 1 (HIF-1) is responsible for the transcriptional activation of the VEGF gene in hypoxic cells by binding to a hypoxia response element (HRE) located 1kb upstream of the transcription initiation site. In addition, the abundance of VEGF mRNA is increased due to stabilization of the mRNA by binding of HuR to the 3' UTR (untranslated region). Under hypoxic conditions, cap-dependent translation is replaced by cap-independent translation of the VEGF mRNA which is mediated by an internal ribosome entry site (IRES) within the VEGF 5'UTR.

[0435] This Example demonstrates the generation of stable cell lines, harboring VEGF 5' and 3'UTR sequences, which can be used to identify small molecular weight compounds that inhibit VEGF IRES-dependent translation or modulate VEGF mRNA stability.

7.2. Materials and Methods

7.2.1. Generation of VEGF 5' and 3'UTRs

[0436] The VEGF 5'UTR was generated using PCR from human genomic DNA. The full-length 5'UTR was prepared by the ligation of two separate PCR products (FIG. 1A). The first half of the 5'UTR (designated VEGF 5'UTR2, encompassing nucleotides 1 to 498) was amplified with primer 1 (5'-AAA GTC GAC GTA ATC GCG GAG GCT TGG GGC AGC CGG-3', SEQ ID NO: 69, and primer 2 (5' TTT GCG ACT GGT CAG CTG CGG GAT CCC AAG 3', SEQ ID NO: 70). The second half of the VEGF 5'UTR (designated VEGF 5'UTR1, from nucleotide 337 to 1038, plus the first 45 bp of the VEGF open reading frame) was amplified using primer 3 (5'-AA GTC GAC GTA AGA GCT CCA GAG AGA AGT CGA G-3, SEQ ID NO: 71 and primer 4 (5'-AAA CCC GGG CAG CAA GGC AAG GCT CCA ATG CAC-3', SEQ ID NO: 72). Each PCR product was digested with BamH I, and ligated together to produce the full length 5'UTR. To facilitate downstream cloning into dicistronic plasmid p2luc-i, primers 1 and 3 were designed to include a Sal I site and a stop code (TAA), immediately after the Sal I site at the 5' end, and primer 4 for VEGF 5'UTR1 includes a Xma I site at the 5' end (FIG. 1C).

[0437] The entire VEGF 3' UTR (shown in FIG. 1B) was amplified by genomic PCR using primer 5 (5'-GCC GGG CAG GAG GAA GGA GCC TCC CTC AGG GTT TCG GGA 3', SEQ ID NO: 73) and primer 6 (5'-CTG CAC TAG AGA CAA AGA CG T GAT GTT AAT -3', SEQ ID NO: 74. The BgI II and EcoR I restriction sites were used for subsequent cloning.

7.2.2. Plasmid Construction

[0438] Each PCR fragment (VEGF 5'UTR1, VEGF 5'UTR2 and VEGF 3'UTR) was cloned into pT-Adv vector for confirmation by DNA sequencing using the Clontech advantage cloning kit. A SalI-XmaI VEGF 5'UTR1 fragment was subcloned into the p2luc-i dicistronic plasmid (FIG. 2A, Grentzmann et al., 1998, RNA 4:479-486). The sequence of the polylinker site is GAA CAA ATG TCG ACG GGG GCC CCT AGC AGA TCT AGC GCT GGA TCC CCC GGG GAG CTC AUG GAA GAC (SEQ ID NO: 75, FIG. 2A). The resulting plasmid (designated p2luc/VEGF5UTR1, see FIG. 2B) contains VEGF 5'UTR1 between the two reporter genes (renilla luciferase and firefly luciferase) with a stop code (TAA) immediately after the Sal I site and a fusion translation junction between the first 15 AA of VEGF and firefly luciferase open reading frame. To construct the dicistronic plasmid containing the full length VEGF 5'UTR, VEGF 5'UTR2 was then subcloned into p2luc/VEGF5UTR1 between SalI and BamHI (designated p2luc/VEGF5UTR-fl; FIG. 2B). This plasmid also has a stop code (TAA) immediately after the Sal I site to prevent read-through from the first reporter to the second.

[0439] To map the region of the IRES essential for activity, dicistronic plasmids containing various deletions within the VEGF 5'UTR were prepared (FIG. 2B). Plasmid p2luc/vegf5'utr-delta51-476 is derived from p2luc/vegf5'utr-fl by removing the Nhe I fragment (nt51 to 746); plasmid p2luc/vegf5utr-delta476-1038 was derived from p2luc/vegf5utr-fl by removing the sequence from BamH I site to the 3'end of 5'UTR; plasmid p2luc/vegf5utr-delta1-476 was derived from p2luc/vegf5utr-fl by removing the sequence from BamH I to the 5'end of 5'UTR.

[0440] To generate stable cell lines for high throughput screening, a monocistronic reporter plasmid (pluc/VEGF5'+3'UTR) containing the VEGF 5' and 3'UTRs and firefly luciferase gene (FIG. 3A) was constructed. Briefly, a Sal I-Not I fragment, containing the full length VEGF 5'UTR and firefly reporter gene, from p2luc/VEGF5'UTR-fl was subcloned into pCDNA5/TO between EcoR V and Not I, and then the VEGF 3'UTR was subcloned into the intermediate plasmid at the Not I site by blunt-end ligation.

7.2.3. DNA Transfection and Generation of Stable Cell Lines

[0441] 293T cells were transfected with pluc/VEGF5'+3'UTR using the Fugene 6 transfection reagent (Roche) according to manufacture's instruction. 48 hours after transfection, the cells were lysed and plasmid function was monitored by measuring luciferase activity using Promega's luciferase kit according to manufacture's instruction.

[0442] To generate stable cell lines, plasmid pluc/VEGF5'+3'UTR was transfected into 293T cells as described. 48 hours after transfection, the cells were trypsinized, resuspended

in culture media plus 200 mg/ml hygromycin B, then seeded in 96 well plates at 100 to 500 cells per well for selection. The media containing hygromycin B was changed every 3 to 4 days. After 10 to 14 days of selection, hygromycin resistant clones were screened under a microscope and wells harboring a single colony were expanded under hygromycin selection for further experiments.

7.2.4. Luciferase Assay

[0443] F. luciferase and R. luciferase activities were measured using the Luciferase reporter assay system (Promega) according to manufacturer's instruction.

7.2.5. Semi-quantitative PCR

[0444] DNA and RNA were isolated from B9 cells using TRIzol reagent (GIBCO BRL) according to the manufacturer's instructions. cDNA was synthesized using Promega's reverse transcription system. Semi-quantitative PCR was performed with gene specific primers for firefly luciferase or glyceraldehyde phosphodehydrogenase (GAPDH) as an internal control. The primer pairs for firefly luciferase amplification were as follows: 5'-CGG TGT TGG GCG CGT TAT TTA TCG GAG TTG-3' (SEQ ID NO: 76) and 5'-TTG GCG AAG AAT GAA AAT AGG GTT GGT ACT-3' (SEQ ID NO: 77); the primer pairs for GAPDH were as follows: 5'-GGT GAA GGT CGG AGT CAA CGG A-3' (SEQ ID NO: 78) and 5'-GAG GGA TCT CGC TCC TGG AAG A-3' (SEQ ID NO: 79). The PCR products were separated on 1% agarose gel, stained with ethidium bromide and quantified on UVP with Labworks software.

7.2.6. High Throughput Screening

[0445] High throughput screening ("HTS") for compounds that inhibit untranslated region-dependent expression of vascular endothelial growth factor ("VEGF") is accomplished using stable cell lines described in Section 7.2.3. The 293T cell line contained stably integrated copies of the firefly luciferase gene flanked by both the 5' and 3' UTRs of VEGF. Cell lines exhibiting consistently high levels of firefly luciferase expression are further expanded and optimized for HTS.

[0446] Screening of compounds is done using one hundred 384-well plates per day. Each 384-well plate contains a standard puromycin titration curve that is used as a reference to calculate % inhibition and the statistical significance of the data points generated in the assay. This curve occurs in wells from column 3 and 4 of the 384-well plate. The concentration of puromycin is 20 mM serially diluted 2-fold to 0.078 mM plated in quadruplicate. Columns 1 and 2 contain 16 standards each of a positive control 0.5% DMSO and a negative control consisting of the puromycin at 20 mM. The difference

between the two controls is used as the window to calculate the percentage of inhibition of luciferase expression in the presence of a compound. Columns 5 through 24 contain compounds from a library of small molecules.

[0447] Two confluent T175 flasks of the VEGF stable cell line described above (B9) are split into twenty T175 flasks three days prior to screening. On each day of the HTS assay, the cells are dislodged from the flask with 3 ml of 0.25 % trypsin-EDTA (Gibco, cat no. 25200-056) and diluted to 10 ml with non-selective media. This is repeated for all twenty flasks and the cells are combined, counted and diluted to a concentration of 263.15 cells/ml. 38 to 39 ml are then added to each well containing 1 to 2 ml of compound from a small molecule library to a final compound concentration of 7.5 mM (3.75 mg/ml) in 0.5 % DMSO. The puromycin standard curve also contains 0.5 % DMSO. The stable cell line is incubated in the presence of compound overnight (approximately 16 hours) at 37 C in 5 % CO_2. To monitor firefly luciferase activity, LucLite Plus (Packard cat no. 6016969) is prepared according to manufactures' instructions and 20 ml is added to each well. Following a brief incubation at room temperature (minimum 2 min.), firefly luciferase activity in each well is detected with the ViewLux 1430 ultraHTS Microplate Imager (Perkin Elmer). All data obtained is uploaded into Activity Base for % inhibition calculations and statistical analyses.

7.3. Results

[0448] The ability of VEGF 5'UTR sequences to modulate internal translation initiation was tested using the plasmid vector that encodes a dicistronic mRNA (FIG. 2A). The renilla luciferase is translated from the first cistron by a cap-dependent scanning mechanism, while the firefly luciferase in the second cistron is translated only if preceded by an internal ribosome entry site. In this study, five discistronic plasmids containing various deletions of the VEGF 5'UTR (FIG. 2B) were generated and transiently transfected into 293T cells to monitor IRES-dependent translation of firefly luciferase. 48 hours after transfection, extracts were prepared and assayed for renilla and firefly luciferase activities using the dual luciferase kit from Promega. As shown in FIG. 2C, deletion of either the first 336 or the first 476 nucleotides has no significant effect on firefly luciferase activity compared to full length VEGF5'UTR directed luciferase levels. However, deletion between nucleotides 51 and 746 decreased firefly luciferase activity more than 75% (33.68+/-4.91 vs 161+/-30.49). Deletion of nucleotides 476 to the 3' end of the VEGF 5'UTR decreased firefly luciferase activity more than 90% (12.15+/-1.2 v.s. 161+/-30.49). Taken together, these results confirm that the VEGF 5'UTR harbors IRES activity, and also

indicates that the region of the VEGF IRES essential for function is located within nucleotide 476 to the 3' end of VEGF 5'UTR.

[0449] To generate stable cell lines for High Throughput Screening ("HTS"), a monocistronic reporter plasmid under the transcriptional control of the CMV promoter (pluc/vegf5'+3'UTR; FIG. 3A) was constructed. This plasmid contains both the VEGF 5'- and 3'-UTRs separated by the firefly luciferase gene. After confirmation of luciferase production by transient transfection (data not shown), transfected 293-T cells were seeded in 96 well plates at a concentration of 100-500 cells per well, and then cultured under hygomycin B selection. After two weeks of selection, 19 clones were screened for luciferase activity, three of which demonstrated high levels of luciferase activities (clones B9, D3, H6; FIG. 3B). To determine which cell line demonstrated the highest level of expression, the luciferase activities of clones B9, D3, H6 were compared and normalized against the protein concentrations extracted from each cell line. The results shown in FIG. 4 demonstrate that the luciferase activity from B9 cells was two fold greater than H6 cells, and more than three fold higher than D3 cells.

[0450] To determine if the B9 cells are stable, these cells were maintained under hygromycin selection for more than three months, with intermittent monitoring of luciferase activity. The results indicate that this cell line is stable and sustains a high level of luciferase expression when continuously cultured in vitro for more than three months (FIG. 5). Sustained expression of luciferase by B9 cells indicated that the monocistronic plasmid integrated into the genomic DNA. Semi-quantitative PCR was performed to determine the number of copies of the reporter plasmid integrated per B9 cell. As FIG. 6A shows, series diluted plasmid pluc5'+3'vegf-UTR were included as positive control to make sure the reaction for sample (genomic DNA from B9 cells) was within the linear range, i.e., not saturated. The PCR standard curve was plotted with the PCR product intensity against the amount of positive plasmid control loaded for PCR (FIG. 6B). Sigma plot regression indicated that PCR product intensity for B9 genomic DNA (50 ng) is about the same level of 6.4 pg plasmid control. As 1 mg of 8 kb plasmid roughly contains 10^{11} copies and 10^{6} cells have 10 mg genomic DNA, the results here indicated that approximately 100 copies of the plasmid were integrated per cell.

[0451] High throughput screening ("HTS") for compounds that inhibit untranslated region-dependent expression of vascular endothelial growth factor ("VEGF") is accomplished with the generated stable cell lines.

8.3. Results

[0466] To determine the effect of the survivin untranslated regions on post-transcriptional control of gene expression, transient transfections of the survivin expression vectors described in Section 8.1.2., containing both, one or none of the 5' and 3' UTRs of survivin both in the absence or presence of the stem-loop secondary structure were performed. In the absence of the stem-loop secondary structure, cap-dependent and cap-independent translation are equally favored and no significant difference in firefly luciferase expression could be detected when either or both of the 5' and 3' UTRs are present or absent (FIG. 7A). This results confirms the earlier notion that, in the survivin expression vectors without the stem-loop secondary structure, the 5' UTR of survivin is unable to block cap-dependent translation. In the presence of the stem-loop secondary structure, a 3-fold increase in firefly luciferase expression can be detected only in the survivin expression vectors that contain the 5' UTR of survivin (FIG. 7B). This result strongly suggests that the 5' UTR of survivin can function as an internal ribosome entry site and promote cap-independent translation and helps explain the increase in the endogenous levels of survivin in the G2/M phase of the cell cycle when overall translation is dramatically reduced.

[0467] High throughput screening ("HTS") for compounds that inhibit untranslated region-dependent expression of survivin is accomplished with the generated stable cell lines.

9. EXAMPLE: HER-2

[0468] This Example demonstrates the generation of stable cell lines, harboring Her-2 5' and 3'UTR sequences, to identify small molecular weight compounds that inhibit Her-2 5' UTR-dependent translation or modulate Her-2 mRNA stability.

9.1. Her-2 Constructs

9.1.1. Generation of Her-2 in vitro Expression Constructs

[0469] The 99 nucleotide 5' UTR of Her-2 was PCR-amplified from a human genomic DNA (Promega) using the following primers: Sense/HindIII: CAAGAAGCTTgcgcccggccccccaccccteg (SEQ ID NO: 86) and Antisense/NcoI: AGCCCATGGtgctcactgcggctccggcccc (SEQ ID NO: 87). The Advantage-GC2-PCR kit was used according to the manufacturer's instructions (Clontech) with the following conditions: PCR cycle conditions were 94 C, 3 minutes, followed by 35 cycles of 94 C, 30 seconds, and 68 C, 30 seconds. The PCR-amplified product was cloned using the pT Adv kit (Clontech) according to the manufacturer's instructions. All clones were confirmed by

sequencing. The resulting clone was digested with HindIII/NcoI and the fragment was cloned into pT7Luc, upstream of the luciferase gene, to generate pT7Luc/5'UTR.

[0470] The 615 nucleotide 3'UTR was PCR-amplified from human genomic DNA (Promega) using the following primers: sense/BglII: agactctgaaccagaaggccaa (SEQ ID NO: 88) and antisense/KpnI: ctcggtaccagttttccaaaatatatttgcaaatgg (SEQ ID NO: 89). The Titanium Taq kit (Clontech) was used according to the manufacturer's instructions with the following amplification conditions: 94 C, 1 minute, followed by 35 cycles at 94 C, 30 seconds to denature, 60 C 30 seconds to anneal, 72 C 1 minute to extend. The product was gel purified and cloned using pT Adv (Clontech) according to the manufacturer's instructions. All clones were sequenced. The resulting clone was digested with BglII/KpnI and cloned into a BglII/KpnI digested pT7Luc and pT7Luc/5'UTR to generate pT7Luc/3'UTR and pT7Luc/5'and3'UTR, respectively.

9.1.2. Generation of Her-2 in vivo Expression Constructs

[0471] Constructs for cell-based expression were generated by isolation of Her-2 containing fragments of pT7Luc/5'UTR, pT7Luc/3'UTR and pT7Luc/5' and 3'UTR digested with HindIII and KpnI and cloned into pcDNA (+) (Invitrogen).

9.1.3. Generation of Her-2 uORF Mutants

[0472] The uORF contained within the Her-2 5' UTR was removed by extending the overlapping long primers. The overlapping sequence is underlined. The sense minus uORF HindIII primer is: cccaagcttcgcgcccggccccccaccccctcgcagcaccccgcgccccgcgccctccc (SEQ ID NO: 90) and the antisense minus uORF NcoI primer is: ggccccatggctccggctggacccggctgggacccggctgggagggcgcgggagggcgcgg (SEQ ID NO: 91). The primers (10 micrograms) were denatured at 95 C for 2 minutes, annealed at 60 C for 5 minutes and extended at 72 C for 10 minutes using Taq polymerase (Clontech). After buffer-exchange, the product was digested with NcoI and HindIII and cloned in the HindIII/NcoI sites of the in vitro expression vector pT7Luc and pT7Luc/3'UTR, yielding pT7Luc/5'UTR minus uORF and pT7Luc/5'UTR minus uORF and 3' UTR. Both plasmids were digested with HindIII and KpnI and the Her-2 containing fragment was subcloned into the HindIII/KpnI site of pcDNA (+) (Invitrogen) for cell-based studies.

9.2. Stable Cell Line Production

[0473] Stable cell lines were generated in HeLa, 293T, and MCF-7. First, transient transfection was carried out using the Fugene 6 transfection reagent (Roche) according to manufacturer's instructions. Untranslated region-dependent firefly luciferase activity was

monitored forty-eight hours post-transient transfection with the luciferase reporter assay system (Promega) according to manufacture's instructions.

[0474] Next, stable cell lines were generated by first transiently transfecting the above cell lines. Instead of lysing the transiently transfected 293T cells (or other), the cells were trypsinized, counted and seeded (10 ml) in 10 cm petri dishes at a concentration of 5000 cells/ml. The following day, hygromycin B was added in culture media to a final concentration of 100 mg/ml for 293T cells and 200 mg/ml for MCF-7 and Hela, to select for cells in which the transiently- transfected plasmid has stably integrated into the genome. Following ten to fourteen days of hygromycin B selection, individual hygromycin-resistent clones were expanded by transferring the cells from the petri dish to a single well in a six or twenty-four well plate using trypsin-soaked filter discs according to manufacture's instructions. Individual cell lines are then selected for further studies based on firefly luciferase expression levels.

9.3. *In vitro* High Throughput Screen

[0475] Construct pT7Luc/5'and 3'UTR is utilized as a template for large-scale T7 polymerase transcription according to the manufacturer's protocol (Ambion). The mRNA template containing the Her-2 5' and 3' UTR and the Luciferase ORF is uncapped and used at 100 nanograms/reaction for a typical in vitro HTS. The number of samples run determines the amount of transcription yield that must be obtained. For example, for 100,000 reactions, using 100 nanograms of RNA/reaction, 10 milligrams of RNA must be produced. Typical yields from the Ambion T7 Transcription Kit for this template are 5 mg/ml of transcription.

[0476] Screening of compounds is done using one hundred 384-well plates per day. Each 384-well plate contains a standard puromycin titration curve that is used as a reference to calculate % inhibition and the statistical significance of the data points generated in the assay. This curve occurs in wells from column 3 and 4 of the 384-well plate. The concentration of puromycin is 20 mM serially diluted 2-fold to 0.078 mM plated in quadruplicate. Columns 1 and 2 contain 16 standards each of a positive control of 4% DMSO and a negative control consisting of the puromycin at 20 mM. The difference between the two controls is used as the window to calculate the percentage of inhibition of luciferase expression in the presence of a compound. Columns 5 through 24 contain compounds from a library of small molecules.

[0477] The *in vitro* translation reaction of the Her-2 driven Luciferase ORF consists of four microliters of rabbit reticulocyte lysate (Green Hectares) supplemented with 0.013 mgs/ml hemin (Sigma), 0.05 mgs/ml creatine kinase (Roche), and 0.125 mgs/ml tRNA

(Sigma Type XII, rabbit liver), 100 nanograms uncapped mRNA and buffer containing 100 mM KOAc, 0.5 mM $Mg(OAc)_2$, 10 mM creatine phosphate, 0.03 mM amino acid mix, in a reaction volume of 20 ml. The reaction is then incubated at 30 C for 45 minutes. At the end of incubation, 20 mL of LucLite (Packard) is added to the reaction and the light output resulting from luciferase catalyzed conversion of luciferin, is monitored on a ViewLux uHTS Plate reader (Perkin Elmer).

10. EXAMPLE: CELL EXPRESSION VECTORS

[0478] Stable cell line expression vectors (pCMR1 and pCMR2) are shown in FIGS. 8A and 8C. pCMR1, a high-level stable and transient mammalian expression vector designed to randomly integrate into the genome and pCMR2 is an episomal mammalian expression vector. pMCP1 (FIG. 8B) is a high level stable and transient mammalian expression vector designed to site-specifically integrate into the genome of cells genetically engineered to contain the FRT site-specific recombination site via the Flp recombinase (see, *e.g.*, Craig, 1988, Ann. Rev. Genet. 22: 77-105; and Sauer, 1994, Curr. Opin. Biotechnol. 5: 521-527). The nucleotide sequences are presented below.

[0479] pCMR1 (SEQ ID NO: 92)

gacggatcgggagatctcccgatcccctatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagtatctgctccct
gcttgtgtgttggaggtcgctgagtagtgcgcgagcaaaatttaagctacaacaaggcaaggcttgaccgacaattgcatgaagaat
ctgcttagggttaggcgttttgcgctgcttcgcgatgtacgggccagatatacgcgttgacattgattattgactagttattaatagtaatc
aattacggggtcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaac
gacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtggagtattt
acggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgc
ctggcattatgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatcgctattaccatggtgatgcgg
ttttggcagtacatcaatgggcgtggatagcggtttgactcacggggatttccaagtctccaccccattgacgtcaatgggagtttgttt
tggcaccaaaatcaacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtacggtggga
ggtctatataagcagagctctctggctaactaagctttcggcgcgccgaggtaccatgggatccgaagacgccaaaaacataaaga
aaggcccggcgccattctatcctctagaggatggaaccgctggagagcaactgcataaggctatgaagagatacgccctggttcct
ggaacaattgcttttacagatgcacatatcgaggtgaacatcacgtacgcggaatacttcgaaatgtccgttcggttggcagaagctat
gaaacgatatgggctgaatacaaatcacagaatcgtcgtatgcagtgaaaactctcttcaattctttatgccggtgttgggcgcgttattt
atcggagttgcagttgcgcccgcgaacgacatttataatgaacgtgaattgctcaacagtatgaacatttcgcagcctaccgtagtgtt
tgtttccaaaaaggggttgcaaaaaattttgaacgtgcaaaaaaaattaccaataatccagaaaattattatcatggattctaaaacgga
ttaccagggatttcagtcgatgtacacgttcgtcacatctcatctacctcccggttttaatgaatacgattttgtaccagagtcctttgatcg
tgacaaaacaattgcactgataatgaattcctctggatctactgggttacctaagggtgtggcccttcgcatagaactgcctgcgtca
gattctcgcatgccagagatcctatttttggcaatcaaatcattccggatactgcgattttaagtgttgttccattccatcacggttttggaa
tgtttactacactcggatatttgatatgtggatttcgagtcgtcttaatgtatagatttgaagaagagctgtttttacgatcccttcaggatta

WHAT IS CLAIMED IS:

1. A method for identifying a compound that modulates untranslated region-dependent expression of a vascular endothelial growth factor (VEGF) gene, said method comprising:

(a) contacting a member of a library of compounds with a cell containing a nucleic acid comprising a reporter gene operably linked to an UTR of the VEGF gene; and

(b) detecting a reporter protein translated from said reporter gene, wherein a compound that modulates untranslated region-dependent expression of a VEGF gene is identified if the expression of said reporter gene in the presence of a compound is altered as compared to the expression of said reporter gene in the absence of said compound or the presence of a control.

2. A method for identifying a compound that modulates untranslated region-dependent expression of a VEGF gene, said method comprising:

(a) contacting a member of a library of compounds with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to an UTR of the VEGF gene; and

(b) detecting the expression of said reporter gene, wherein a compound that modulates untranslated region-dependent expression of a VEGF gene is identified if the expression of said reporter gene in the presence of a compound is altered as compared to the expression of said reporter gene in the absence of said compound or the presence of a control.

3. The method of claim 1 or 2, wherein the UTR of the VEGF gene is the 5' untranslated region (5' UTR) of a VEGF gene.

4. The method of claim 3 wherein the 5' UTR of the VEGF gene is operably linked upstream of the reporter gene.

5. The method of claim 1 or 2, wherein the UTR of VEGF gene is the 3' untranslated region (3' UTR) of a VEGF gene.

6. The method of claim 5,wherein the 3' UTR of the VEGF gene is operably linked downstream of the reporter gene.

7. The method of claim 3,wherein the nucleic acid further comprises the 3' UTR of a VEGF gene.

8. The method of claim 7,wherein the 3' UTR of the VEGF gene is operably linked downstream of the reporter gene.

9. The method of claim 1 or 2, wherein the reporter gene further comprises an intron.

10. The method of claim 1 or 2, wherein the UTR of the VEGF gene comprises an iron response element ("IRE"), internal ribosome entry site ("IRES"), upstream open reading frame ("uORF"), or AU-rich element ("ARE").

11. The method of claim 1 or 2, wherein the nucleic acid is further polyadenylated at the 3' end.

12. The method of claim 1 or 2, wherein the nucleic acid is not capped at the 5' end.

13. The method of claim 1 or 2, wherein the reporter gene encodes firefly luciferase, renilla luciferase, click beetle luciferase, green fluorescent protein, yellow fluorescent protein, red fluorescent protein, cyan fluorescent protein, blue fluorescent protein, beta-galactosidase, beta-glucoronidase, beta-lactamase, chloramphenicol acetyltransferase, or alkaline phosphatase.

14. The method of claim 1, wherein said cell is stably transfected with said nucleic acid.

15. The method of claim 1, wherein said cell is transiently transfected with said nucleic acid.

16. The method of claim 1, wherein said cell is transfected with an episomal expression vector comprising said nucleic acid.

17. The method of claim 1 or 2 further comprising measuring the effect of said compound on the expression of the VEGF gene.

18. The method of claim 1, wherein the cell is a human cell, a yeast cell, a mouse cell, a rat cell, a Chinese hamster ovary ("CHO") cell, a MCF-7 cell, a primary cell, or an undifferentiated cancer cell.

19. The method of claim 18 wherein the human cell is a HeLa cell or a 293 cell.

20. The method of claim 3, wherein the cell-free translation mixture is a cell extract.

21. The method of claim 20, wherein the cell extract is derived from is a human cell, a yeast cell, a mouse cell, a rat cell, a Chinese hamster ovary ("CHO") cell, a Xenopus oocyte, a MCF-7 cell, a primary cell, an undifferentiated cancer cell, a reticulocyte, or a rye embryo.

22. The method of claim 1 or 2, wherein the compound is selected from a combinatorial library of compounds comprising peptoids, random biooligomers, diversomers, vinylogous polypeptides, nonpeptidal peptidomimetics, oligocarbamates, peptidyl phosphonates, peptide nucleic acid libraries, antibody libraries, carbohydrate libraries, and small organic molecule libraries.

23. The method of claim 22, wherein the small organic molecule libraries are libraries of benzodiazepines, isoprenoids, thiazolidinones, metathiazanones, pyrrolidines, morpholino compounds, or diazepindiones.

24. The method of claim 1, wherein the step of contacting a library of compounds with a cell is in an aqueous solution comprising a buffer and a combination of salts.

25. The method of claim 24, wherein the aqueous solution approximates or mimics physiologic conditions.

26. The method of claim 24, wherein the aqueous solution further comprises a detergent or a surfactant.

27. The method of claim 1 or 2 further comprising (c) determining the structure of the compound that modulates untranslated region-dependent expression of the VEGF gene.

28. The method of claim 27, wherein the structure of the compound is determined by mass spectroscopy, NMR, vibrational spectroscopy, or X-ray crystallography.

29. The method of claim 1 or 2, wherein the compound directly binds to an RNA transcribed from the VEGF gene.

30. The method of claim 1 or 2, wherein the compound binds to one or more proteins that modulate untranslated region-dependent expression of the VEGF gene.

31. The method of claim 7, wherein the compound disrupts an interaction between the 5' UTR and the 3' UTR of the VEGF gene.

32. A method of modulating the expression of a VEGF gene comprising contacting a cell expressing the VEGF gene with a compound, or a pharmaceutically acceptable salt thereof, identified according the method of claim 1 or 2.

33. A method of treating, preventing or ameliorating cancer or one or more symptoms thereof, said method comprising administering to a subject in need thereof an effective amount of a compound, or a pharmaceutically acceptable salt thereof, identified according to the method of claim 1 or 2, wherein said effective amount decreases the expression of the VEGF gene.

34. A method of inhibiting or reducing angiogenesis, said method comprising administering to a subject in need thereof a prophylactically or therapeutically effective amount of a compound, or a pharmaceutically acceptable salt thereof, identified according to the method of claim 1 or 2, wherein said effective amount decreases the expression of the VEGF gene.

35. A method of identifying a compound that inhibits or reduces angiogenesis, said method comprising:

(a) contacting a member of a library of compounds with a cell containing a nucleic acid comprising a reporter gene operably linked to an UTR of a VEGF gene; and

(b) detecting the expression of said reporter gene, wherein if a compound that reduces the expression of said reporter gene relative to the expression of said reporter gene in the absence of said compound or the presence of a control is detected in (b), then

(c) contacting the compound with a tumor cell and detecting the proliferation of said tumor cell, so that if the compound reduces or inhibits the proliferation of the tumor cell, the compound is identified as a compound that inhibits or reduces angiogenesis.

36. The method of claim 38 further comprising (d) testing said compound in an animal model for angiogenesis, wherein said testing comprises administering said compound to said animal model and verifying that angiogenesis is inhibited by said compound in said animal model.

37. A method of treating, preventing or ameliorating cancer or one or more symptoms thereof, said method comprising administering to a subject in need thereof an effective amount of a compound, or a pharmaceutically acceptable salt thereof, identified according to the method of claim 35.

38. A method of inhibiting or reducing angiogenesis, said method comprising administering to a subject in need thereof an effective amount of a compound, or a pharmaceutically acceptable salt thereof, identified according to the method of claim 35.

39. A method of identifying a compound that inhibits or reduces angiogenesis, said method comprising:

(a) contacting a member of a library of compounds with a cell-free translation mixture and a nucleic acid comprising a reporter gene operably linked to an UTR of a VEGF gene; and

(b) detecting the expression of said reporter gene, wherein if a compound that reduces the expression of said reporter gene relative to the expression of said reporter gene in the absence of said compound or the presence of a control is detected in (b), then

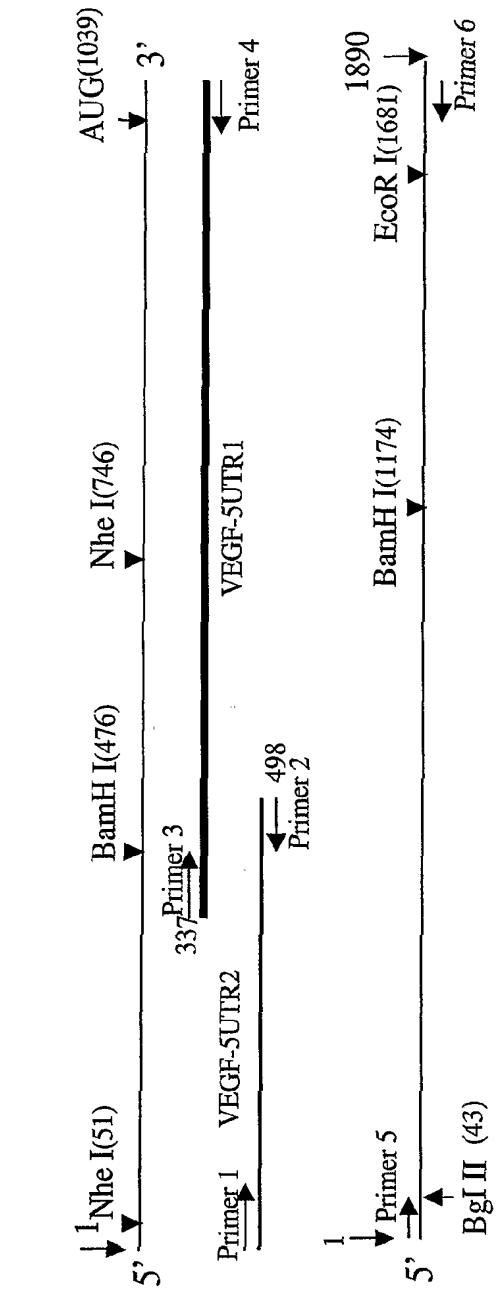
(c) contacting the compound with a tumor cell and detecting the proliferation of said tumor cell, so that if the compound reduces or inhibits the proliferation of the tumor cell, the compound is identified as a compound that inhibits or reduces angiogenesis.

40. The method of claim 39 further comprising (d) testing said compound in an animal model for angiogenesis, wherein said testing comprises administering said compound to said animal model and verifying that angiogenesis is inhibited by said compound in said animal model.

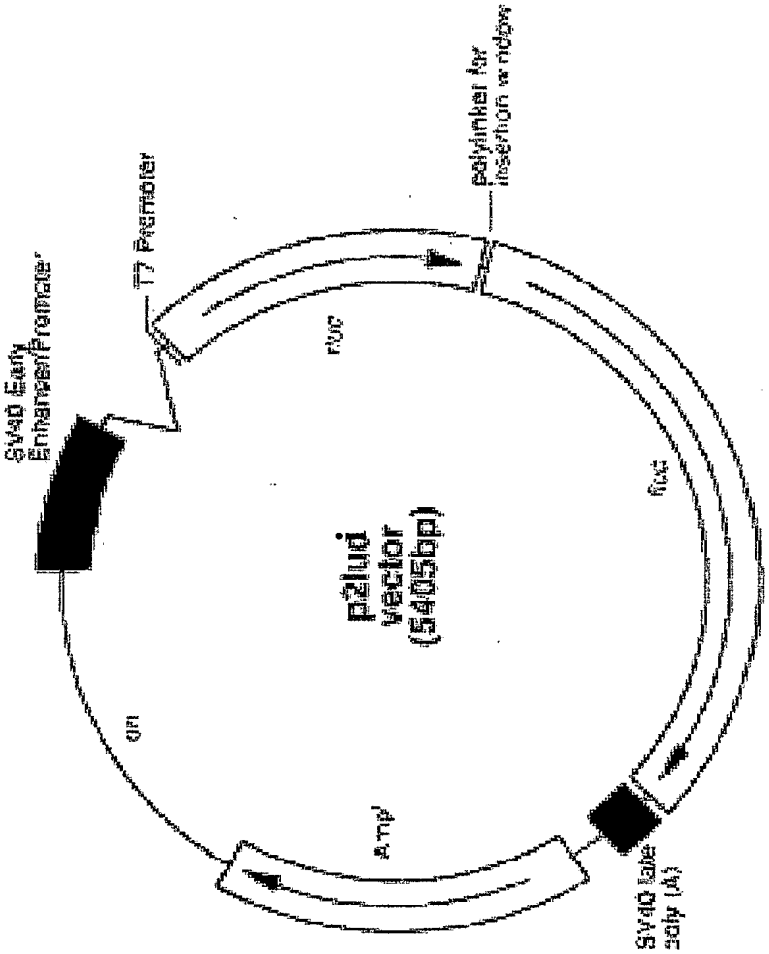
41. A method of inhibiting or reducing angiogenesis, said method comprising administering to a subject in need thereof an effective amount of a compound, or a pharmaceutically acceptable salt thereof, identified according to the method of claim 39.

42. A method of treating, preventing or ameliorating cancer or one or more symptoms thereof, said method comprising administering to a subject in need thereof an effective amount of a compound, or a pharmaceutically acceptable salt thereof, identified according to the method of claim 39.

43. The method of claim 1 or 2 further comprising determining the specificity of the compound for the VEGF untranslated region.



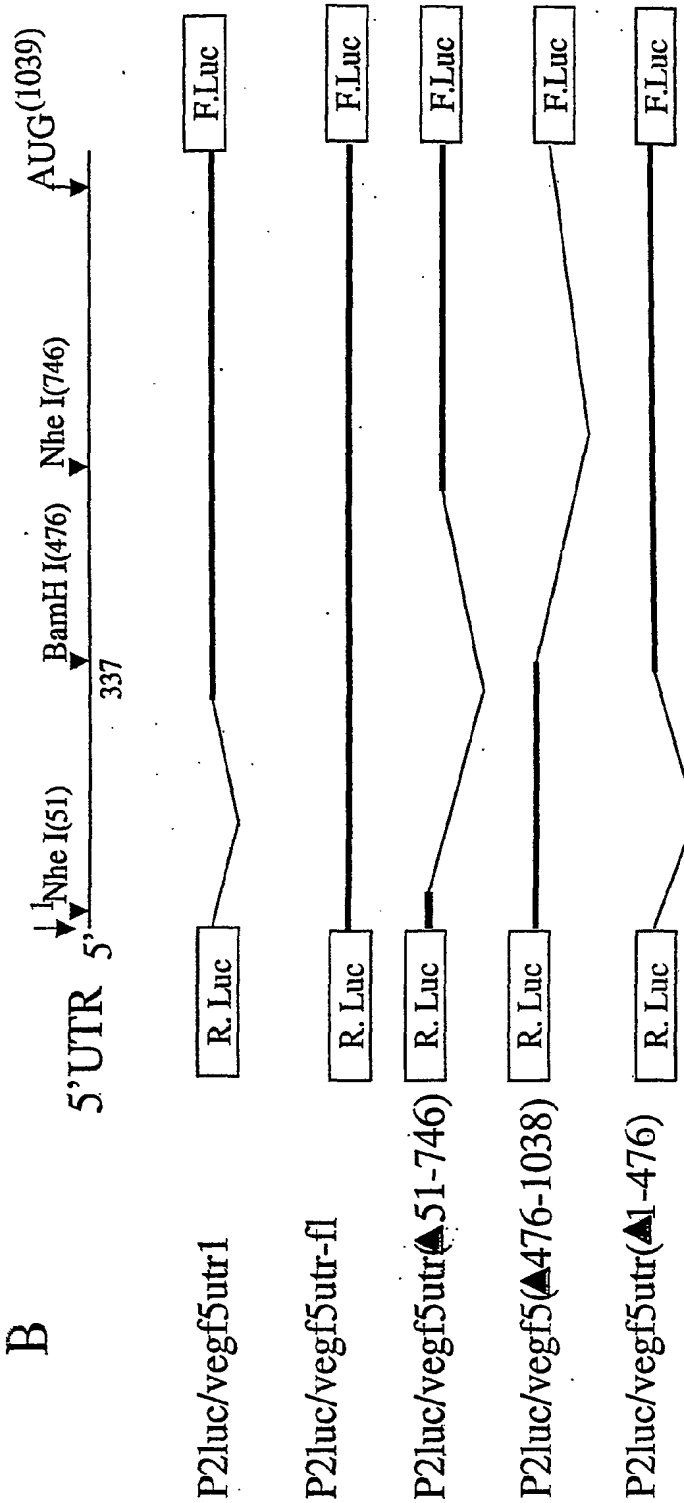
FIGURES 1A TO 1C

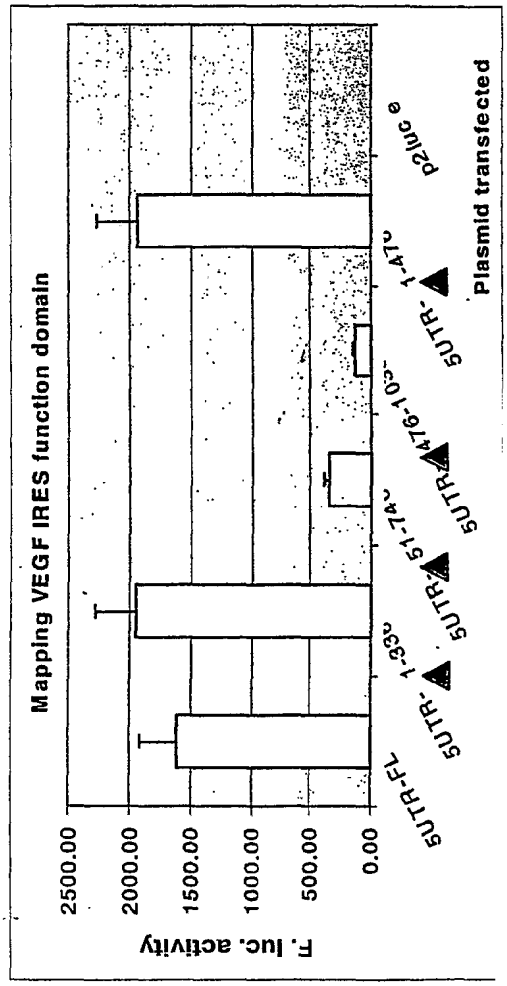


Polylinker sites

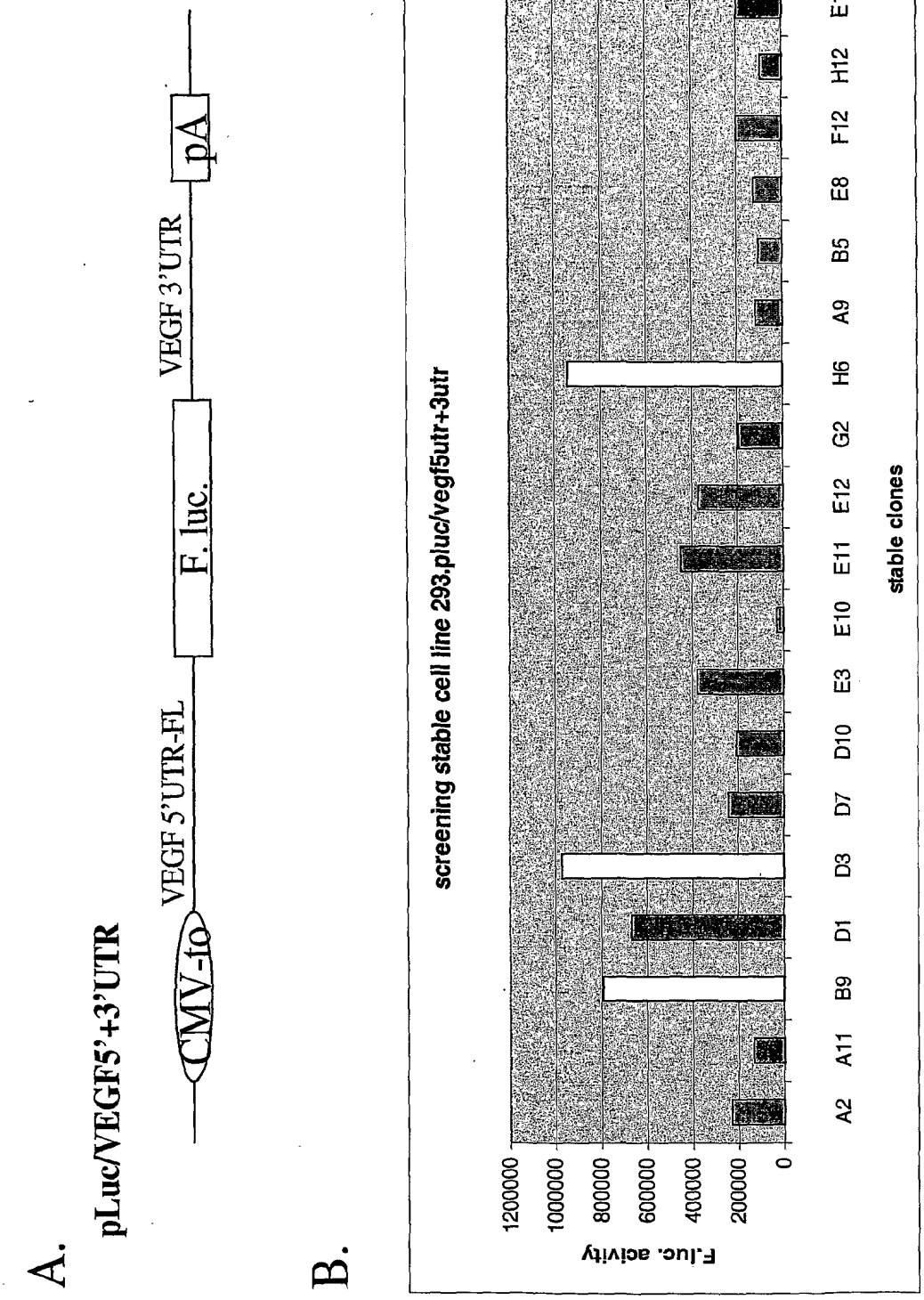
p2luci SalI ApaI EglII Bsu47III BamHI SmaI SacI
GAA CAA ATG TCG ACG GGC CCC CTT AGG AGA TCT AGC GCT GGA TCC CCC GGG GAG CTC AUG GAA GAC
RLuc. M S M E Fluc

FIGURE 2A





FIGURES 2B TO 2C



FIGURES 3A TO 3B

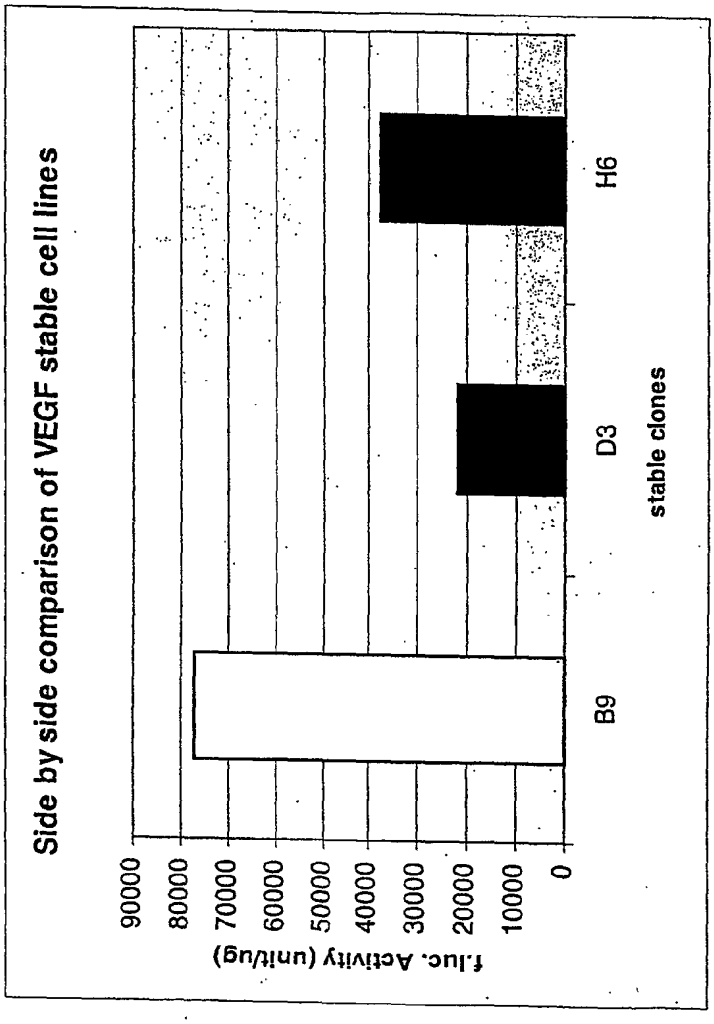

Side by side comparison of VEGF stable cell lines
f.luc. Activity (unit/ug)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
B9
D3
H6
stable clones

FIGURE 4

Stability of stable cell line B9

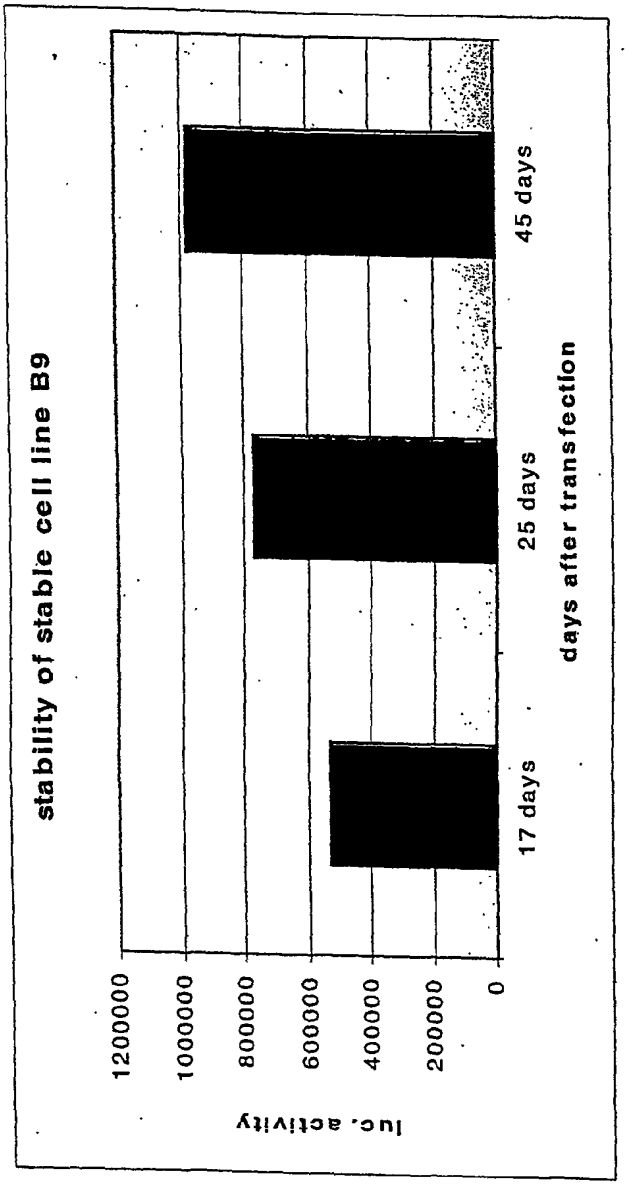
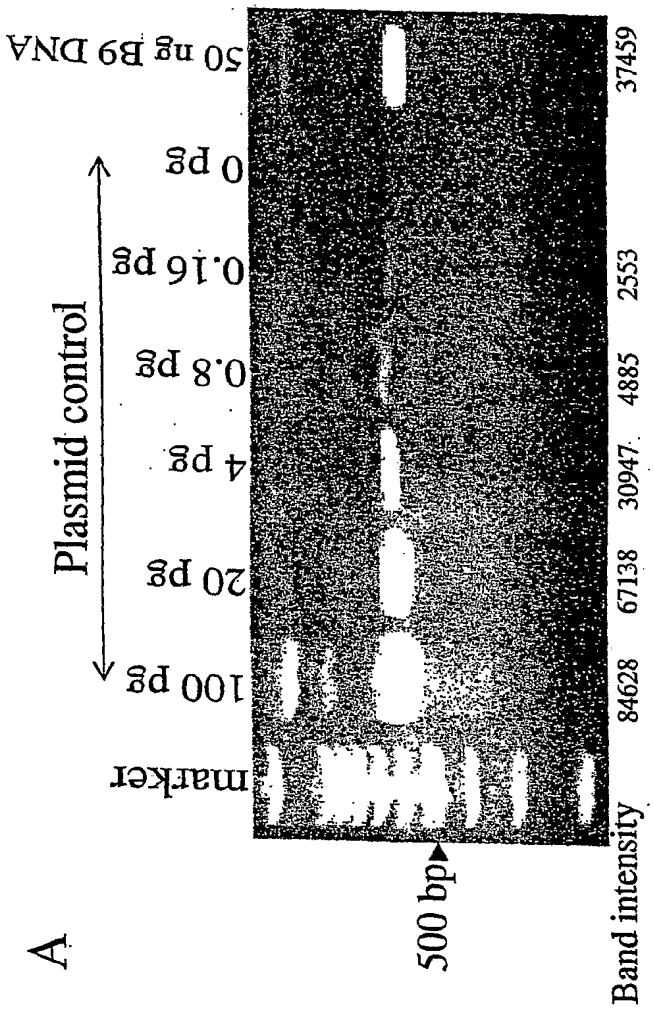
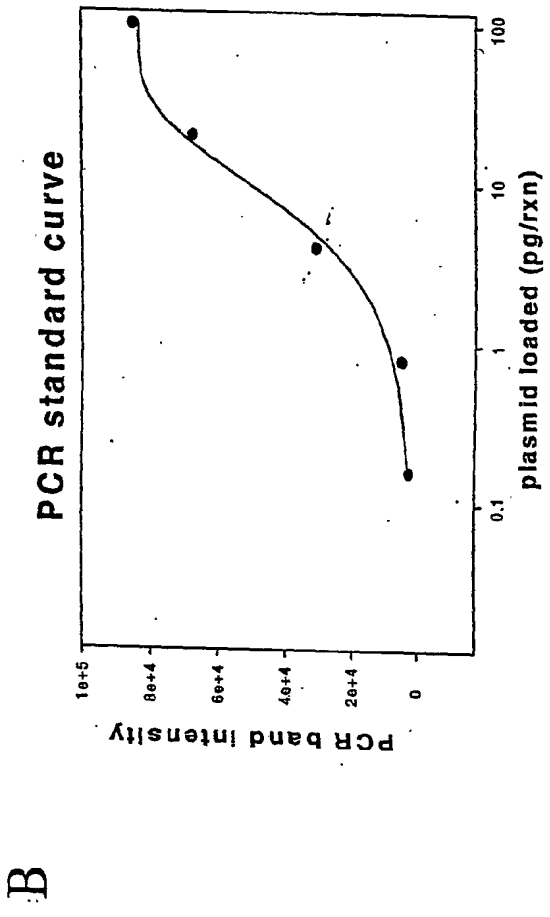
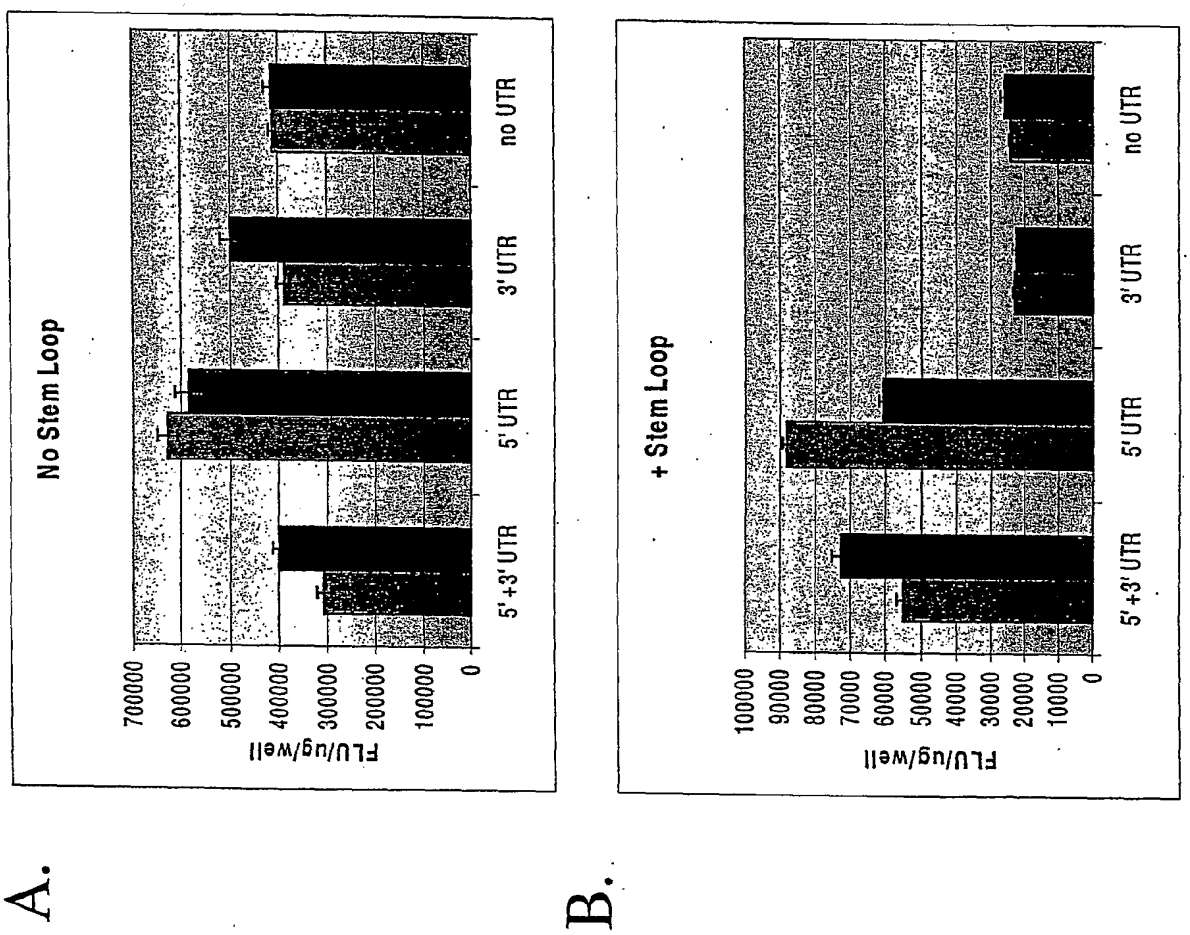


FIGURE 5





FIGURES 6A TO 6B



FIGURES 7A TO 7B

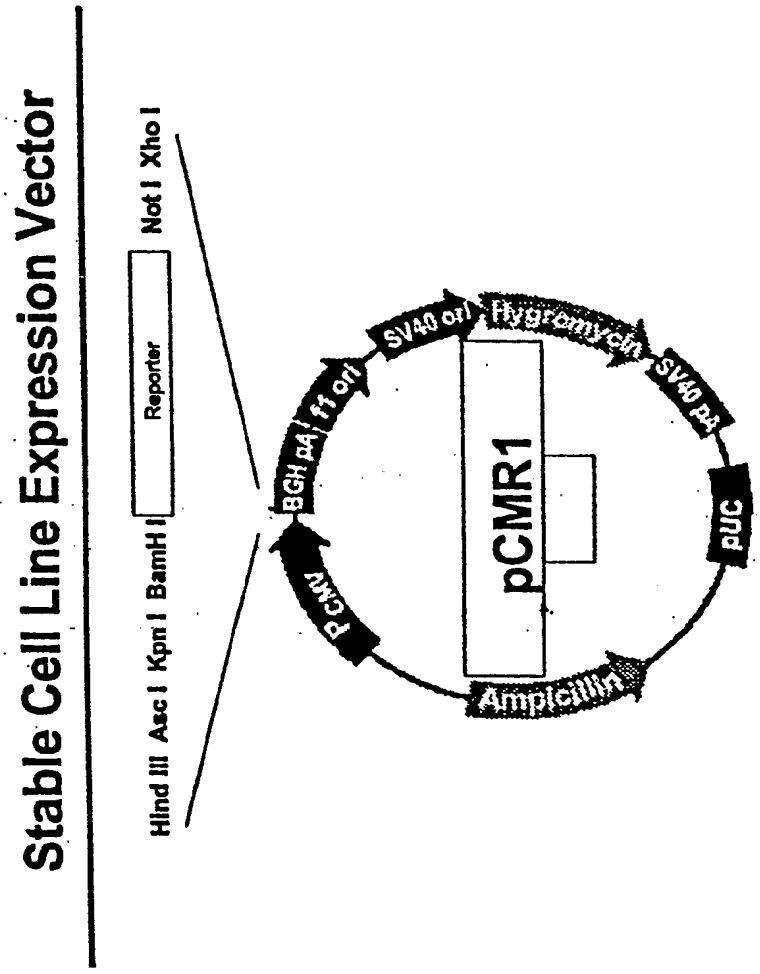

Stable Cell Line Expression Vector
Hind III Asc I Kpn I BamH I
Reporter
Not I Xho I
pCMR1
PCMV
BGH pA
f1 ori
SV40 ori
Hygromycin
SV40 pA
pUC
Ampicillin

FIGURE 8A

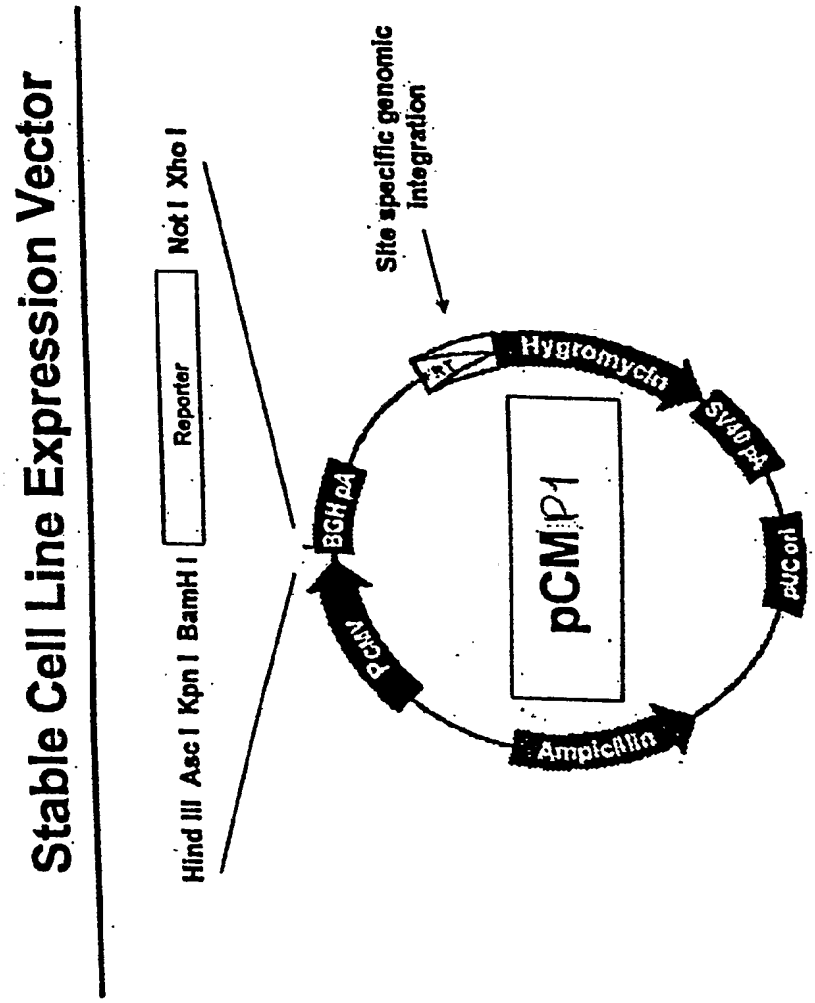

Stable Cell Line Expression Vector
Not I Xho I
Reporter
Hind III Asc I Kpn I BamH I
Site specific genomic integration
pCMIP1
Hygromycin
SV40 PA
pUC ori
Ampicillin
PCMV
BGH pA

FIGURE 8B

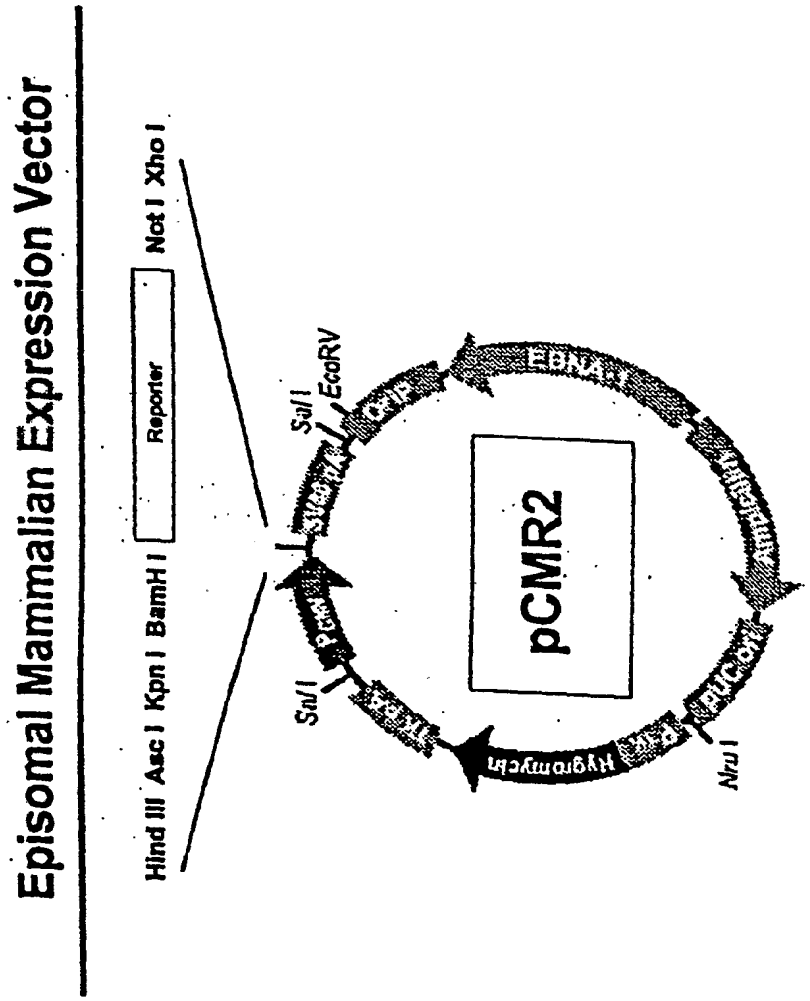

Episomal Mammalian Expression Vector
Hind III Asc I Kpn I BamH I
Reporter
Not I Xho I
Sal I EcoRV
pCMR2
EBNA-1
Hygromycin
Sal I
Nru I

FIGURE 8C